ADENOVIRUS INDUCED ADENO-ASSOCIATED VIRUS GENE EXPRESSION IS NOT DEPENDENT ON AAV NON-STRUCTURAL REP PROTEIN

By

DANIEL FRANCIS LACKNER

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This work is dedicated to both of my parents, John and Sally Lackner.

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Daniel Francis Lackner

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The normal lifecycle of Adeno-Associated Virus (AAV) alternates between a latent, repressed provirus and an actively transcribing viral genome. This switch in gene expression is the combination of cellular and viral factors to promote AAV replication. Previous studies of AAV have indicated regulatory pathways, which maintain the correct AAV transcription levels for productive infection. Autoregulation of the AAV genome is controlled by Rep protein interactions with cellular proteins. The addition of Adenovirus helper activities induce changes in both cellular and AAV transcriptional regulation. This combination of regulatory factors allows the dissection of a complex transcriptional regulation mechanism in a relatively simple genetic system. The dissection of the AAV transcriptional regulation was performed by a series of promoter constructs that would access the factors required for transcriptional activation of the AAV p19 promoter. Initially, the first promoter constructs containing a single AAV Rep protein binding site were used to determine if the nonstructural Rep protein would function as a

transcriptional activator of AAV gene expression. The Rep protein was determined not to be a transcriptional activator but instead to function as a position-dependent inhibitor of p19 promoter activity. This position-dependent inhibition of the AAV p19 promoter activity suggested that the previously characterized Rep and Sp1 interaction might not be the direct cause of Rep-mediated transactivation. In order to prove that the previous characterized Rep and Sp1 interaction was not responsible for induction of the p19 promoter, a novel system of hybrid transcription factors was used to replace the Rep and Sp1 proteins. The novel hybrid transcription factors modified from the yeast two-hybrid system, consisted of two different proteins containing a GAL4-DNA binding domain fused to either an interaction domain from p53 or T-antigen. The replacement of the Rep and Sp1 binding sites with GAL4 binding sequences in the p19 promoter showed that Rep and Sp1 were not required for transactivation. We conclude that the Rep and Sp1 transcription factors function as architectural proteins facilitating a DNA loop to form between the Rep binding site in the p5 promoter and the Sp1 site within the p19 promoter.

CHAPTER 1
INTRODUCTION

Eukaryotic Gene Expression

The regulation of eukaryotic gene expression has been shown to be far more complex than the original protein fractionations for RNA polymerase activities suggested. Initial fractionation protocols discovered three distinct RNA polymerases and their respective General or Basal Transcription Factors (GTFs). However, purification of these factors did not restore the complex regulation of transcription in vitro indicating that more factors were required for proper transcriptional regulation. More recent studies have observed that the RNA polymerase II holoenzyme (RNAP II) is about the same size as the ribosome and many other factors are critical for correct gene expression. DNA condensation into chromatin has been shown to regulate gene expression and the exposure of DNA sequences allows the assembly of transcriptional regulators to recruit the complete RNA polymerase II holoenzyme.

The Chromatin Template

The DNA template of most promoters for protein-encoding genes contains three important features. The first component is the transcription initiation site that directs the initiation of polymerization of mRNA, which is catalyzed by the RNA polymerase II holoenzyme. The second component is the TATA box which regulates the binding of the TATA binding protein (TBP). The last component is the regulatory sequences

surrounding the transcription initiation site and the TATA box of the expressed gene product.

The transcription initiation site and the TATA box are defined as the core promoter elements. This core element averages about 100 base pairs (bp) in length for most protein encoding genes. The AT-rich sequence named the TATA box is usually found about 25 to 30 bp upstream of the transcription initiation site in higher eukaryotic organisms (179). The TATA box was initially characterized for its ability to bind the TATA binding protein (TBP), a component of the TFIID complex that is important for initiating the formation of the RNA polymerase II holoenzyme. The binding activity of this AT-rich region was named for its apparently canonical sequence of TATA but it has been recently observed that TBP can bind to a range of sequences no longer defined by a simple sequence match to the TATA box region (58, 170, 208). In addition to the more common TATA box, many promoters can have an initiator element (Inr) that covers the transcriptional start site and recruits regulatory factors and the RNA polymerase II (171, 172). The core promoter can therefore comprise a TATA and Inr elements (composite), either element alone (TATA- or Inr-directed), or neither element (null) (135). Most viral-encoded proteins have composite promoters, most cellular RNAP II genes are TATA-directed promoters, and many null promoters have many different transcriptional initiator sites reflecting variable TBP binding positions (51, 79, 111).

The majority of gene regulation on protein-encoding genes functions at the level of upstream activating sequences, upstream repressing sequences, silencers, or locus control regions. Upstream activating sequences (UAS) are defined as sequences of DNA that bind specific transcription factors that promote the recruitment of the RNAP II at the

core promoter element and activate the initiation of gene expression. These activating sequences can be relatively close to the initiation site which allows activators to bind near the core promoter, thus defining this subgroup of UASs as proximal promoter elements (129, 179). Another subgroup of UASs, defined as enhancers, consist of binding sites for one or more transcriptional activators that function independent of their orientation and can be separated by DNA sequences of 85 kilobasepairs (kb) (15). Upstream repressing sequences (URSs) are DNA sequence elements that function negatively toward the recruitment and assembly of the RNA polymerase II holoenzyme at the nearby proximal core promoter element. In contrast to active recruitment by activators, the repressive elements that bind to URSs function in preventing gene expression by different mechanisms. The first mechanism is the passive exclusion of activator binding, thereby preventing the activator from RNA pol II recruitment. Most commonly, the repressor will have a binding site sequence that is nearby or overlaps the activator, thereby excluding its binding by steric interference of their binding sites. Another active repressor mechanism is the modification of chromatin structure by histone deacetylation, which prevents transcriptional activators from binding their respective DNA sequences.

Silencers are defined as DNA sequence elements that repress promoter activity in an orientation- and position-independent manner (136). The silencer elements contain binding sites that recognize specific protein factors. These protein factors bind the silencer element and interact with chromatin histones H3 and H4. The result of this interaction is a deacetylation of the H3 and H4 histones inducing a condensation of the chromatin structure, thus preventing access and binding of any transcriptional activators.

Locus control regions (LCRs) are positive activators of expression for proximal genes but differ from upstream activating sequences in several aspects. After these LCR elements are integrated into cellular chromatin, the enhancer sequences function in an orientation and distance independent manner but the level of gene expression is influenced by the actual site of integration. This effect of integration is defined as position effect variegation because the structure of chromatin can overwhelm the enhancer's effect and the accessibility of transcriptional activators to the enhancer sequence. In contrast to enhancer elements, the locus control regions can always activate transcription when integrated into cellular chromatin because the LCR elements directly interact with the formation of the chromatin structure (44, 49, 55).

The last major chromatin element is the Insulator element which can limit the effects of the other regulator elements. The Insulator elements allow the chromatin template to be subdivided into different functional domains. Each chromatin domain is regulated by various enhancers, UASs, URSs, or LCRs and those respective elements are separated not by distance but by the function of the Insulator elements (10, 11, 182).

Chromatin structure is comprised of a series of nucleoprotein complexes called nucleosomes. A nucleosome contains 146 bp of genomic DNA wrapped around an octomer of histone proteins (92, 226). The octomer is arranged in a cylinder shape composed of two heterodimers of histones H3 and H4 and another two heterodimers of histones H2A and H2B. The nucleosome crystal structure shows DNA wrapped around the cylinder and the contact is maintained by interactions with the histone proteins and the phosphate backbone of the DNA (116). The DNA is bound so that the N-terminus of the histones can interact with other neighboring nucleosomes to form higher order

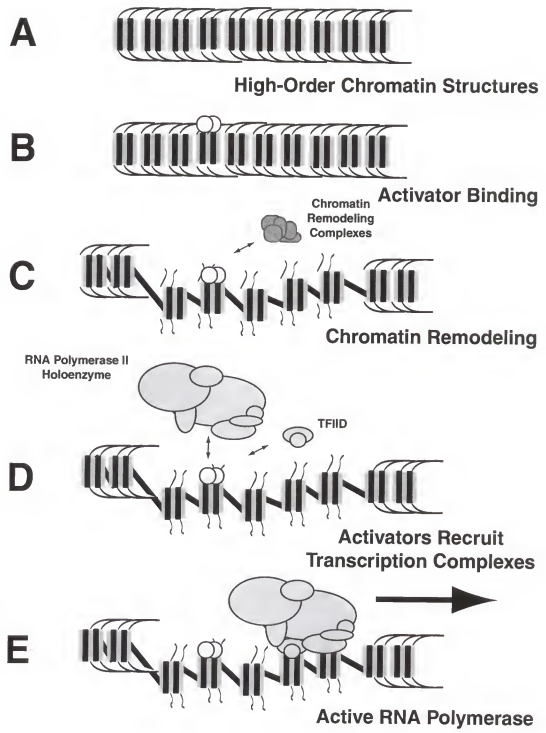
structures. In addition, the DNA and histone interactions are not fixed allowing some promoter sequences to be exposed for gene expression. The degree of chromatin structure and condensation within a promoter region is usually related to the level of transcriptional activation (Fig. 1, A) (56, 142, 152, 212). Many in vitro experiments have observed that transcriptional templates preincubated with chromatin fractions inhibit the initiation of RNA polymerases. In complementing experiments, in vivo yeast experiments which depleted histone proteins increase the transcriptional activity of many different genes (60, 61, 214). These experiments demonstrate how tight histone-DNA contacts and the organization of nucleosomes into higher order structures can limit and sterically inhibit binding of transcription factors to the promoter region.

Activation and Repression

An activator is composed of two different functional protein domains (187). The first domain binds the specific DNA sequence within the upstream activating sequence and the second domain functions to recruit or stimulate the activity of the RNA polymerase II holoenzyme. A single activator can be used in order to activate multiple genes throughout the genome allowing synchronization of many different gene products. On the other hand, a single gene in the genome can be regulated by many separate transcriptional activators providing the genome a mechanism for variable control.

The main function of transcriptional activators is the binding and recruitment of the transcription initiation apparatus (Fig. 1, B) (151). This function has been demonstrated by direct binding between the activation domains of transcription factors and components of the transcription machinery (3, 59). Further evidence for the role of activation domains in binding and recruiting the transcription apparatus is provided by replacing the activation domains with an artificial activator such as a fusion protein

Fig. 1. **A model for the role of activators in transcriptional initiation.** *A*, Genomic DNA is packaged into nucleosomes an higher ordered chromatin structures. *B*, Transcriptional activators bind to promoter sequences and can have a positive influence on promoter activity. *C*, Activators bind and recruit chromatin remodeling and modifying complexes that influence local chromatin structure. *D*, Activators bind and recruit the transcriptional initiation apparatus to promoters through the interactions of a few large complexes. *E*, Activators can affect the promoter clearance and RNA Polymerase II holoenzyme processivity.


A
High-Order Chromatin Structures
B
Activator Binding
Chromatin Remodeling Complexes
C
Chromatin Remodeling
RNA Polymerase II Holoenzyme
TFIID
D
Activators Recruit Transcription Complexes
E
Active RNA Polymerase

between a DNA binding domain and corresponding domains from the transcriptional machinery. This artificial activator can substitute for the actual activator in vivo by exhibiting the same functional role in transcriptional activation (5, 26, 215).

Repressors of transcriptional activity usually function in either a general activity or in a very gene-specific manner (105). The general repressors of transcription interact with the TBP. In yeast, Mot1 represses transcription of some genes by directly binding the TBP-DNA complex and inducing dissociation of TBP from the TATA-box sequence (2, 131, 194). In large eukaryotic organisms, the Dr1 protein is another general negative regulator of RNA polymerase II transcription. The Dr1 protein binds the basic repeat domain of TBP bound to promoter DNA and prevents the assembly of the RNA polymerase II holoenzyme (53, 80, 85).

Gene-specific repressors can function by direct physical interaction with transcriptional activators or by competition for activator binding sites. One classic model of gene-specific repression is the GAL4 and GAL80 interaction in yeast. The GAL4 transactivator stimulates the expression of yeast genes with a GAL4 binding site. However, the activity of GAL4 can be inhibited by its direct physical interaction with GAL80 (117). The GAL80 protein represses the GAL4 activator by binding and masking a portion of its activation domain (169). Another repressor, chaperone Hsp90, functions by a different mechanism to inhibit the activity of the transcriptional activator Hsf1. Instead of an interaction which masks the activation domain, the Hsp90 protein prevents the formation of Hsf1 trimers required for the binding of the Hsf1 activators to its respective DNA element (227). These are two examples of how direct interaction or inhibition of an activator can lower transcriptional activity in a gene-specific manner.

Another method of gene-specific repression is steric interference at the level of DNA binding activity. In this mechanism, no direct interaction between an activation and a repressor is necessary. Two transcription factor groups representing activator and repressor activities compete for neighboring DNA promoter sequence. An example of this type of regulation is found between the Acr1 repressor and the ATF/CREB activator (193). Promoters that use steric interference for transcriptional regulation have overlapping Acr1 and ATF/CREB sites within their promoter regions.

The interaction between activation and repression is also strongly dependent on the expression and concentration levels of the positive and negative transcription factors. A strong experimental example of this interplay between activation and repression can be found in yeast mutational screens that create loss-of-function alleles in negative transcription factors and are complemented by mutations in positive transcriptional activators (104, 149, 150).

Chromatin Modification

The compression of genomic DNA into nucleosomes and higher order chromatin organization provides yet another level of transcriptional regulation. The interactions between the N-terminus of histone proteins allows many different chromatin complexes to form based on the ability of the histones to contact one another. The majority of this variable chromatin structure is regulated by the aceytlation of the N-terminal domains of histones (130, 188, 225). The acetylation of histones induces the disruption of tight, high-order chromatin structures and increases transcription activity. On the other hand, various factors causing histone deacetylation allows the formation of higher-order chromatin structure and results in the repression of transcription.

Histone proteins are modified by acetylation, phosphorylation, methylation, and ubiquitination. The acetylation modification is the best understood with several lysines on the N-terminus of each histone protein reversibly acetylated (176). After acetylation, the nucleosomes are less likely to undergo physical interactions and condensation so the exposed DNA sequences may allow increased recruitment of the transcriptional machinery (Fig. 1, C) (63, 224). Additionally, acetylation of histones neutralizes the positively charged lysine groups thereby decreasing the histones affinity for DNA (56, 116). Acetylated histones may also directly interact with transcriptional activators increasing their binding affinities and inducing more RNA polymerase II holoenzyme recruitment (212).

Histone acetyltransferases (HATs) were first identified with transcriptional activation by the structure and function studies of the Tetrahymena protein, p55. The p55 protein was known to function as a histone acetyltransferase but later studies showed it was related in sequence to the yeast transcriptional cofactor Gcn5 (16). The relationship between these two proteins correlated the activities of histone acetylation and increased transcriptional activation. For experimental evidence, the Gcn5-dependent promoters were shown to have increased acetylation of histones. Additionally, mutations in the HAT domain inhibited the ability of Gcn5 to activate transcription and acetylate promoter histones (96, 200). Several transcriptional coactivators that bind to activators, such as p300 and CBP, have HAT activity. The HAT activity enables them to alter the chromatin structure of proximal promoters after activators bind to DNA (43, 52, Ogryzko, 1996 #3438). Histone Deacetylases (HDACs) were first correlated to transcriptional repression by the identification of purified HDACs that have similarity to transcriptional

cofactors (156). As more cellular histone deacetylases were isolated, many were found to be components of corepressors recruited by transcription factors.

Basal Transcription Factors

In order for any promoter sequence to function, a number of general transcription factors are required to direct RNA polymerase II to the specific site of transcriptional initiation. The general transcription factors, comprised of TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH, are necessary for assembly of RNA polymerase at the core promoter and for transcription initiation (Fig 1, D) (34). The combination of the RNA polymerase II and the basal transcription factors melts the core promoter sequence to create a open complex in which 12 to 15 basepairs (bp) of DNA unwind to form a single-stranded bubble (33, 54, 189). This open complex allows initiation to occur by catalyzing a number of phosphodiester bonds in messenger RNA (mRNA). In most cases of transcription initiation, RNA polymerase II and associated factors initiate transcription and release small mRNA fragments from the open promoter complex (118). Finally, the RNA polymerase II overcomes these abortive initiations to synthesize longer mRNA which extends past the open promoter complex. After 25 to 30 bp, the elongating complex pauses and usually requires stimulation from transcription factors in the promoter complex and modification of the RNAPII to undergo promoter escape and create the entire mRNA transcript (189).

One of the most critical factors for initiating transcriptional activity is the TATA-Binding Protein. The TBP is similar to a symmetrical molecular saddle on the core promoter DNA (25, 134). When the TBP is bound to a TATA box, the molecular saddle binds the promoter and causes a sharp bend in the DNA strands which induces a partial unwinding of base pairing at the initiation site (84). Along with TBP, the TFIID complex

contains the TBP-associated factors or TAFs (18, 192). The TAFs regulate the ability of the TBP to select the specific site of transcriptional initiation. The requirement for the TAFs is most clearly seen in the difference between the TATA-directed core promoters and the Inr-directed or null core promoter elements. The TBP protein is able to initiate transcription on TATA box elements, independent on any TAF proteins in the TFIID fraction. However, for Inr-dependent promoters or null core promoter sequences, the TAF proteins are required for transcriptional activation and interaction with the transcription factors in the URSs (119).

The TFIIA fraction acts directly with TBP and stabilizes the TBP-DNA interaction (120). The protein subunits within TFIIA have direct contacts with transcriptional activators (140, 220). TFIIA may inhibit general transcriptional repressors by displacing or blocking those proteins from their respective effects on TFIID activity (139). The TFIIB fraction has been shown to directly influence the selection and site of trancriptional activation (12, 146). The TFIIB subunit structure seems to set the distance between the core promoter element and the transcriptional start site. This observation is proven by TFIIB mutants which shift the site of initiation because they no longer allow interactions between RNA Polymerase II and the TFIIB fraction (4, 17, 19). Recent crystal structure experiments have shown that the actual distance between TFIIB and the RNA polymerase II catalytic site is about 32 bp which is the average distance between most core promoter TATA box elements and the transcriptional start site (107). TFIIB is able to interact with a large number of transcriptional activators and the resulting interaction is a measure of the effectiveness for that activator in the upstream activating sequence (110, 213).

The TFIIF fraction functions to stabilize the preinitiation complex on the core promoter element. This stabilization may be the result of altering the DNA topology. Experiments that crosslink the preinitiation complex to the DNA show that the DNA is wrapped in one complete turn around the preinitiation complex (153). TFIIF is required for this tight wrapping that causes stress in the DNA resulting in promoter melting and open complex formation. The TFIIF factors also function as a transcription elongation factor. This elongation activity may be regulated by its DNA wrapping ability or an intrinsic kinase activity (154).

The TFIIE function may be related to a zinc ribbon motif within its protein sequence (95). TFIIE can bind single-stranded regions of DNA and enable melting of the core promoter sequence. From experimental data, the requirement of TFIIE in maintaining an open promoter complex can be overcome by a synthetic, premelted promoter sequence (72, 141). The induction and stability of an open promoter complex is related to the actual promoter sequence and the TFIIE requirement has been shown to vary between specific promoter sequences (158, 185). Other functions of the TFIIE fraction may be linked to the TFIIH fraction. TFIIE assembles into the RNA Polymerase II preinitiation complex before TFIIH and may stimulate TFIIH recruitment or the CTD kinase and ATPase activities of TFIIH.

The TFIIH fraction has been characterized into two separate functional complexes. The first complex encompasses the core TFIIH functions and the second complex acts in a kinase/cyclin function. The core TFIIH contains a DNA-dependent ATPase, an ATP-dependent helicase, and a CTD kinase activity (32). The proteins responsible for these activities, XPB/ERCC3 and XPB/ERCC2, are found within the

nucleotide excision repair complex. The XPB helicase is required for promoter opening in vitro and is critical for the conversion from abortive transcription initiation to transcriptional elongation (42, 57, Bradsher, 2000 #3377, 71, 73). The TFIIH kinase/cyclin activities are found within the Cdk7/cyclin H proteins (155, 162, 167). The role of these proteins is to phosphorylate the RNA polymerase carboxy-terminal domain (CTD) (32). The degree of RNA polymerase II CTD phosphorylation is related to the ability to switch from transcription initiation to elongation.

RNA Polymerase II Holoenzyme

The purified eukaryotic core RNA polymerase II contains between 10 and 12 different subunits that catalyze DNA-dependent RNA synthesis in vitro. However, the lack of general transcription factors prevents any specific core promoter recognition. The largest subunit of the RNA polymerase II has a carboxy-terminal domain (CTD) that contains tandem repeats of a consensus peptide sequence (Tyr-Ser-Pro-Thr-Ser-Pro-Ser) (35, 221). The CTD domain is essential for cell growth and there are 52 repeats of the consensus peptide sequence in the human RNA Polymerase II (1, 6, 223). CTD regulation is controlled by the amount of phosphorylation on the repeat sequences (37). Within transcription initiation complexes, the RNA polymerase II CTD domains are not phosphorylated and appear to recruit the Srb/Mediator complexes to the RNA polymerase (86, 127). The Srb/Mediator complex functions to transmit the signals from the upstream transcriptional activators to the components of the RNA polymerase II and its basal transcription factors.

After the recruitment and assembly of the RNA polymerase II preinitiation complex, the holoenzyme switches from a transcription initiation complex to an elongation complex through the modification of the CTD phosphorylation state. The two

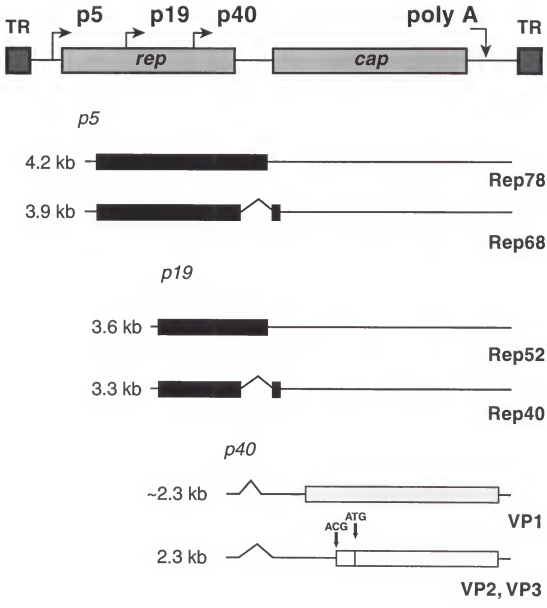
kinases responsible for the CTD phosphorylation are Cdk7 and the Cdk8 proteins (46). The Cdk7 kinase is located within the general transcription factor, TFIIH (115). The TFIIH activity is required for the switch into a stable elongation complex and the Cdk7 kinase phosphorates the CTD subunit allowing release from the initiation complex (42). The Cdk8 protein is a subunit within the Srb/Mediator complex and may regulate transcription elongation through upstream activation factors (Fig. 1, E) (78, 115, 183).

After these two kinases have phosphorylated the RNA polymerase II CTD, the hyperphosphorylated CTD domain promotes the formation of DNA-dependent RNA synthesis along the protein coding sequence. The phosphorylated RNAPII recruits the mRNA capping enzyme to the newly formed transcript and allows mRNA capping to take place soon after promoter clearance (30, 126). In addition to mRNA capping events, the CTD phosphorylation alters the factors and subunits associated with the RNA polymerase II enzyme (137, 208). The Srb/Mediator complex only interacts with the hypophosphorylated CTD and is released with phosphorylation of the CTD. Elongation complexes containing TFIIS are associated with hyperphorylated CTD regions.

Adeno-Associated Virus

Adeno-associated virus (AAV) is a human, single-stranded DNA virus. It has a genome size of 4.7kb that encodes only two open reading frames (ORF), *rep* and *cap* (133). These two ORFs are flanked by two inverted terminal repeats that are required for genome replication, integration, and viral packaging (Fig. 2) (219). An AAV productive infection requires the activities of another virus to alter the cellular replication and transcriptional mechanisms. The most widely characterized helper virus for AAV is

Fig. 2. **Structure of the Adeno-Associated Virus Genome**. In the upper section of the diagram, the two different open reading frames within AAV are shown by rectangles named Rep and Cap. The locations of the p5, p19, and p40 promoters are demonstrated by bent arrows. The terminal repeats (TR) sequences are diagrammed as darkened boxes at both ends of the AAV genome. The lower portion of the diagram shows the AAV transcripts initiated at their relative promoter location. The left side indicates the transcript size and the molecular weight of the protein is on the right.


TR
p5
p19
p40
poly A
TR
rep
cap
p5
4.2 kb
Rep78
3.9 kb
Rep68
p19
3.6 kb
Rep52
3.3 kb
Rep40
p40
~2.3 kb
ACG
ATG
VP1
2.3 kb
VP2, VP3

Adenovirus. Clinically, AAV is only isolated from patients with adenovirus (Ad) infections (13, 14, 148). However, AAV infection alone has never been associated with any disease. If adenovirus is not present during primary AAV infection, an abortive infection results in an integrated proviral structure (28, 125, 128). Most AAV provirus genomes exhibit site-specific integration events in the human genome (93, 160, 165, 204). These genomes can be rescued by superinfection with adenovirus, which induces both proviral rescue and replication (28, 101, 128).

Adenovirus Helper Functions

Several adenovirus genes have been identified that possess specific helper functions during AAV coinfection (48, 132, 133). The adenovirus E1A gene product functions as a trans-activator for both Ad and AAV promoters. The E1A 289 amino-acid protein expressed during an adenovirus infection binds to transcription factors on the AAV p5 promoter and induces AAV gene expression. The E1B 55-kd protein regulates the post-transcriptional export of viral mRNAs into the cytoplasm. The E2 region encodes the Ad DNA binding protein (Ad DBP). The Ad DP promotes transcriptional activation from the AAV promoters and may enhance AAV DNA replication. The E4 region influences many cellular activities to support both Ad and AAV productive infections. The E4 ORF6 gene product increases the degradation of cyclin A and inhibits the kinase activity of Cdc2 that promotes the cell to remain in late S phase of the cell cycle. The induction into S phase will result in the expression of more DNA replication proteins that will convert the single-stranded viral genomes into double-stranded DNA templates Finally, the VA RNAs of Adenovirus enable the initiation of AAV protein synthesis by overcoming Ad interferon-induced host cell shutdown of translation.

AAV Rep Proteins

The AAV genome has three different promoters identified by their map positions of p5, p19, and p40. The p5 and p19 promoters transcribe the *rep* open reading frame. Rep 78 and its alternatively spliced transcript Rep68 are produced from the p5 promoter. The Rep52 and its spliced transcript Rep40 are transcribed from the internal p19 promoter. The larger Rep78/68 proteins have many different activities during AAV infection (9, 64, 67, 70, 97, 98, 100, 123). Rep78 and Rep68 have specific DNA binding activity, site- and strand-specific endonuclease activity, DNA and RNA helicase activity, and an ATPase activity (76, 89, 157, 175, 209). In the absence of adenovirus coinfection, the large Rep proteins repress the AAV promoters (74, 98, 143, 205). However, during Ad infection, Rep78/68 exhibit the dual abilities of transcriptional repression on the p5 promoter and transactivation for the p5, p19, and p40 promoters. Additionally, a model of AAV replication has been proposed in which the Rep78/68 proteins are responsible for nicking the terminal repeats. The site-specific endonuclease activity of the larger Rep proteins nick the terminal repeats thus creating a free 3'OH for AAV genome replication.

In contrast, the smaller Rep52 and Rep40 proteins have fewer characterized activities during AAV replication. In the absence of adenovirus infection, these proteins have the ability to repress AAV promoters while not exhibiting any site specific DNA binding activity (74, 77, 97, 98, 138). Recently, Rep52 has been shown to exhibit ATPase and helicase activity. Additionally, the Rep52/40 proteins have been shown to increase the levels of single-stranded monomer during AAV infection. Since single-stranded genome formation correlates with the packaging of AAV particles, Rep52/40 may function in packaging viral particles (27, 74, 98). Recently, King et al. have

demonstrated that the Rep52 helicase activity is probably directly involved in AAV packaging (87).

AAV Transcription without Adenovirus

The p5 promoter is the most widely studied promoter in the AAV genome because of its direct role in the switch between latent and productive infection in the viral lifecycle. Many previous studies demonstrated that no transcripts are detected during the latent state of AAV (31, 100, 123). The initial studies of the p5 promoter determined that the promoter contained a Major Late Transcription Factor (MLTF) binding site and a previously unknown element which was responsive to the E1A trans-activator of adenovirus. The subsequent cloning of this transcription factor resulted in the identification of the YY1 protein (Ying-Yang protein) named for its dual activities of repression and activation on a downstream promoter (168).

Previous studies in our lab resulted in the identification of another protein binding site in the p5 promoter, the Rep binding element (p5RBE). DNaseI protection and gel shift assays determined that the binding site consisted of a 22 bp sequence (125). Initially, the determination of the p5RBE was performed using purified Rep68 from baculovirus expression vectors. Sequence analysis of the entire AAV genome showed that similar binding sites were located in the A-stem of the terminal repeat as well as the three AAV promoters. The A-stem RBE affinity was shown to be 20 fold higher than the p5RBE while the p19 and p40 binding affinities were 160- and 80- fold less than the A-stem RBE, respectively (125).

AAV Transcription with Adenovirus

Initial analysis of the p19 and p40 promoters was performed in our lab by a novel complementation system (123). Mutational analysis of the two promoters caused direct

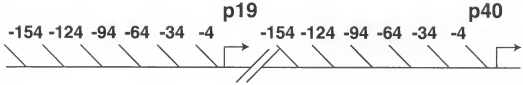
alterations of the Rep protein sequence in the AAV genome. In order to prevent this problem, the Rep protein was supplied in trans by another plasmid p19/40S which contained silent mutations in the Rep protein downstream of the p19 and p40 start sites. To analyze the effects of the deletions within the p19 and p40 promoters, primers were selected that would distinguish between the p19 and p40 mutant promoter transcripts and the transcripts from the Rep complementing plasmid, p19/40S. This allowed the measurement of transcripts by primer extension that were synthesized only from the p19 and p40 promoter deletions.

The deletion analysis of both the p19 and p40 promoters was performed in a series of 5 contiguous 30 bp intervals proximal to each start site. For the p19 promoter, two separated 60 bp regions were necessary for the Rep-mediated activity. One region was identified between positions -153 to -94 and the other was the region from -63 to -4, both upstream of the p19 start site (Fig. 3A). For the p40 promoter, two regions of Rep-mediated induction were identified through deletion analysis. One region was the immediately upstream –60 bp region of p40 while the other region required the -153 to -94 region upstream of the p19 promoter.

Another deletion analysis for both p19 and p40 promoters were performed by progressively larger proximal 5' deletions. The initial deletion named pIM29 dl1-320 eliminated the p5 promoter and resulted in p19 and p40 transcript levels that were 5% of wild type levels. This data revealed a vital element for transactivation of the p19 and p40 promoters that was within the p5 promoter region. Subsequent serial deletions resulted in no greater Rep-mediated induction of p19 or p40 eliminating the possibility of a transcription factor binding site that represses the p19 and p40 promoters (Fig. 3B). In

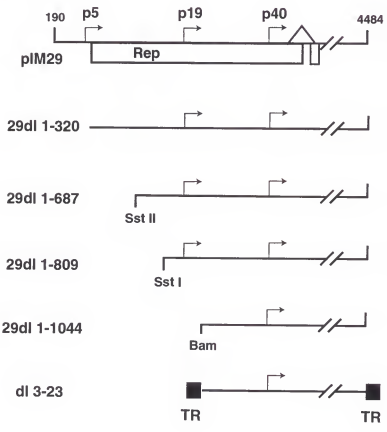
Fig. 3. **AAV Promoters and Promoter Deletion.** *A*, A portion of the plasmid pIM29, containing wild type AAV nucleotides 191 to 4484, is shown with an expanded view of the p19 and p40 promoters. The relative positions of the p19 and p40 promoters are shown by arrows. The locations of the 30bp deletions are diagrammed by lines denoting the nucleotide position relative to the promoter initiation site which precedes each deletion. Below each 30bp deletion, the relative strength of that specific promoter mutant is shown as a precentage of wild type p19 or p40 promoter activity. *B*, The plasmid pIM29 is shown with the Rep coding region shaded. The relative positions of the p5, p19, and p40 promoters are marked by arrows, and the intron is represented by a bent arrow. Below pIM29, the 5'deletions used in that study and the AAV restriction sites used in their construction are shown in the diagram. They are designated by 29*dl* followed by the numbers of AAV nucleotides deleted. The relative transcriptional activity of each promoter mutant plasmid was shown as a percentage of the wild type pIM29 plasmid strength.

A



%wt											
p19	10	10	100	10	< 2		100	100	100	100	100
p40	10	30	150	100	100		92	65	25	10	< 2

B



	% wt	
	p19	p40
pIM29	100	100
29dl 1-320	< 5	5
29dl 1-687	< 5	5
29dl 1-809	5	5
29dl 1-1044	-	5
dl 3-23	-	100

contrast, the last two deletion constructs named pIM29 dl1-1044 and pIM29 dl3-23 differ only by the presence of a terminal repeat. The presence of the terminal repeat caused the Rep-mediated induction of the p40 promoter at wild type levels.

The exact determination of the transcription factors proximal of the p19 promoter was performed by DNase I protection. This biochemical analysis identified the transcription elements SP1-50, GGT-110, SP1-130, and CArG-140 sites relative to the p19 start site (145). Direct determination for some of these protected regions as true SP1 binding sites was performed by competitive gel shifts using SP1 oligos and labeled wt AAV fragments. As a result, the -50, -110, and -130 sites were positively identified as SP1 binding sites upstream of the p19 promoter. The CArG-140 site remained bound during competitive gel shift assays containing a serum response element (SRE) that normally contains authentic CArG-like elements. The next step in the CArG-140 identification procedure was an UV cross-linking experiment, which identified a protein larger than 34kDa. The combination of a CArG-like binding site and a protein mass of 34 kDa indicated a previously uncharacterized protein. After the identification of each binding site, the actual in vivo function for each site on p19 activity was tested using mutants in each transcription factor site. In order to do this in vivo, the p19/40S complementation assay was used to compare the effect of Rep-mediated induction on each p19 promoter mutant. The average p19 transcript level using the wt AAV genome without terminal repeats (pIM45) complemented with p19/40S was normalized to one. The ratio of p19 transcripts in each of the p19 promoter mutants with or without Rep+ p19/40S was calculated to determine which elements were responsible for Rep-mediated induction. If the ratio was relatively unaffected, the p19 element would be vital for Rep-

mediated induction of p19. From this data, only two sites were greatly affected by the Rep+ p19/40S induction of the p19 promoter. These two sites were the SP1-50 and CArG-140. Each resulted in the reduction of p19 transcripts to 20% of wt p19 promoter levels.

Analysis of the p40 promoter by Dnase I protection assay determined a number of protected elements. These elements were identified as AP1-40, SP1-50, GGT-70, and MLTF-100 binding sites. Two additional biochemical assays, competitive gel shift analysis and UV cross-linking, were performed to confirm the identity of the sites.. The combination of these two procedures determined that the SP1-50, GGT-70, and MLTF-100 sites did have transcription factor binding activities. To test the Rep-mediated response of the p40 promoter *in vivo*, transcription factor binding mutants were produced for each site. Using the p19/40S complementation assay, Rep induction depended only on the SP -50 and TATA -30 binding sites. Additionally, the p40 activity was dependent on the CArG-140 element of p19 correlating directly with the previous -153 to –94 bp p40 promoter deletion mutant (144).

This extensive deletion analysis determined that the Rep-mediated induction of both the p19 and p40 promoters required three separate elements. One required region is the proximal SP1-50 element at or in front of each promoter. Another element is a common -153 to -94 p19 region defined as the CArG-140 site. The third and final element appears to be a RBE that can be located in either the left terminal repeat's A-stem RBE or the p5RBE.

To distinguish between the effects of either the p5RBE or the A-stem RBE on the AAV promoters, a series of RBE mutants were created to define Rep-mediated elements

responsible for trans-activation of the p5, p19 and p40 promoters. The AAV genomic subclone lacking terminal repeats (pIM45) was normalized to one for transcript levels from each promoter. The first mutant targeted the p5RBE. As result, the p5 promoter transcription increased by 3.2-fold while the p19 and p40 promoters decreased by 5-fold. This result indicated the p5RBE had a repressive effect on the p5 promoter while simultaneously transactivating the p19 and p40 promoters. The next RBE construct had an intact, wild type p5RBE and a wild type A-stem RBE resembling the fully functional AAV terminal repeat. This construct restored the p19 and p40 transcripts to pIM45 levels while raising the p5 promoter to 3-fold. The final construct had a wild type A-stem RBE and a mutated p5RBE. As a result, this construct exhibited a 8-fold increase in the level of p5 transcripts relative to the pIM45 subclone. Additionally, the p19 and p40 promoters had a 2-fold increase in transcription compared to pIM45 levels. This last construct showed the p5RBE had a direct repressive effect on p5 transcription while the more distal A-stem RBE transactivated the p5 promoter. However, the p5RBE and the A-stem RBE appear to be equivalent and redundant transcriptional elements in Rep-mediated transactivation of the p19 and p40 promoters. During adenovirus infection, Rep 78 or Rep 68 appeared to behave as a transcription factor that can be both a repressor (of p5) and a transactivator (of p19 and p40).

Analysis of the Rep binding abilities on both the terminal repeat RBE and the p5RBE show that either the Rep78 or Rep68 proteins were required for trans-activation of the three AAV promoters. The Rep78 and Rep68 proteins were known to interact but no analysis had been performed on the ability of the larger Reps to bind either Rep52/40 or any cellular proteins. The initial analysis for large and small Rep interaction was

performed using immunoprecipitation against the larger Rep protein domains and by detecting any Rep proteins with an antibody against the common Rep protein carboxy-terminal end. From this immunoprecipitation, the larger Rep proteins were only able to precipitate the Rep52 protein. The in vivo interaction between the large Rep78 and 68 proteins and Rep52 indicated a method of interaction between the Rep78/68 binding element within the TR and the p5RBE, the large Rep proteins, and the smaller Rep52 protein. To detect Rep78/68 effects on the p5RBE, a p5 promoter CAT construct was co-transfected with a constant amount of Rep78/68-expressing plasmid and increasing amounts of a Rep52 plasmid. The result of this titration was the derepression of the p5 promoter through an increasing gradient of Rep52 concentration.

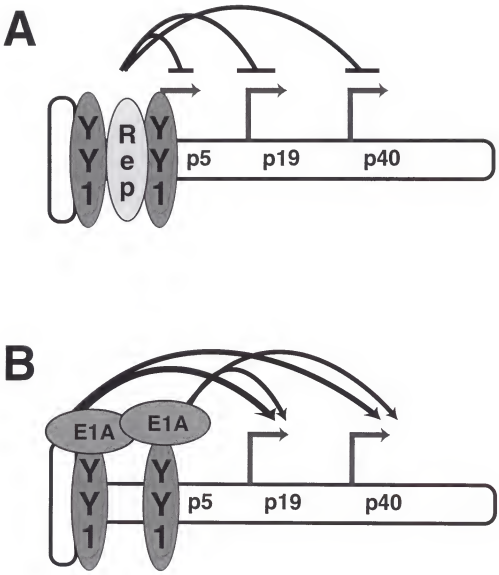
Another suspected Rep78/68 interaction was the common SP1-50 binding site that is conserved within the parvovirus family. The initial analysis of a Rep78/68 and SP1 interaction was performed using a gel supershift assay with an Sp1-50 binding site. The positive supershifting gel fragment indicated a direct role for the Rep78/68 interaction with Sp1. Trans-activation of the p19 and p40 promoters could result from the Rep78/68 bound to the upstream RBEs and each of the SP1-50 binding sites proximal to each promoter. In order to test a model for DNA looping, an AAV genome fragment containing the p5RBE and the SP1-50 p19 was incubated with purified Rep68 and SP1 proteins. The mixture was examined by electron microscopy and analyzed by digitized measurements. These measurements showed a conserved DNA looping structure correlating to the direct interaction between the large Rep proteins bound at the p5RBE and the Sp1 protein at the p19 -50 binding site.

Model for Activation of an AAV Productive Infection from Latency

For this model, the proviral structure of AAV is limited to one proximal terminal repeat containing a single A-stem RBE site. The cellular YY1 and MLTF transcription factors are bound to their sites within the p5 promoter. In the absence of adenovirus infection, YY1 bound at the -60 position of the p5 promoter acts as strong repressor of Rep78/68 transcription (Fig. 4, A) (168). The specific activity of the YY1 -60 binding prevents any trans-activation of the p5, p19, or p40 promoters through either the terminal repeat or the p5RBE.

During adenovirus infection, the Ad early proteins are responsible for the switch between AAV latency and productive infection. The E1A trans-activating protein provides the first helper function for AAV transcription. The E1A protein has three conserved domains for interaction with cellular cell cycle control proteins. The E1A CR1 and CR2 regions bind the pRB and p107 proteins. Expression of the adenovirus E1A protein prevents E2F:pRB complex formation. E2F displacement allows the protein to act as a transcription factor for many cellular replication proteins. However, the CR3 region of E1A interacts with the p300 protein in infected cells. This physical interaction between E1A and p300 is important because the YY1 repressor protein has been shown previously to bind p300. The last 17 amino acids of YY1 are responsible for this interaction and YY1 binding mutants are unable to show an E1A-mediated response. As a result, the E1A protein binds specifically with a p300:YY1 complex on the p5 promoter (24, 103, 108, 168). This binding activity induces a conformational change upon the YY1 protein in a repressive state to expose its activation domain. This change induces p5 promoter trans-activation (Fig. 4, B). After the exposure of the -60 YY1 activation domain, p5 transcription occurs through a mechanism involving the YY1 initiator

Fig. 4. **Loss of AAV Promoter Repression through Adenovirus Infection.** *A*, In the absence of Adenovirus infection, YY1 and Rep proteins bound at the p5 promoter act as strong repressors of AAV gene expression. *B*, during Adenovirus infection, the Ad E1A protein binds to the YY1 proteins and converts their function as repressors to activators of AAV gene expression.


A
YY1
Rep
YY1
p5
p19
p40
B
E1A
E1A
YY1
YY1
p5
p19
p40

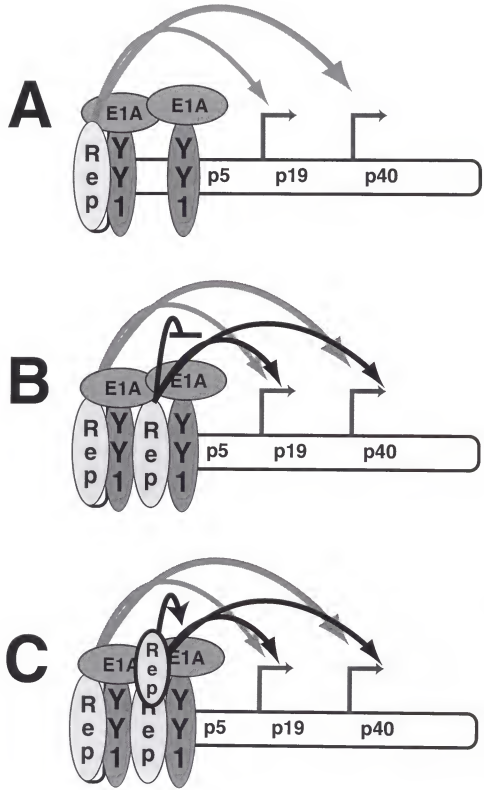
element. The YY1 initiator element binds to DNA and interacts with the TFIIB complex. The YY1 protein is stabilized by its TFIIB interaction facilitating pre-initiation complex formation at the p5 promoter (191).

Induction of the p5 promoter results in the increased cellular concentration of Rep78/68. These two proteins bind the RBEs within the AAV genome as a direct function of their increased concentrations. The first RBE bound is the terminal repeat A-stem. The binding of Rep 78 and/or Rep 68 trans-activates the p5, p19, and p40 promoters. As a direct result, the levels of larger Reps are increased through transcriptional activation (Fig. 5, A). In addition, the Rep 52/40 and capsid proteins are increased by the A-stem RBE trans-activation upon the p19 and p40 promoters. Rep68 is shown to specifically bind the p5RBE and trans-activate the p19 and p40 promoters. After p5RBE binding, the p5 transcription levels decrease while p19 and p40 transcription levels increase (Fig. 5, B).

As the relative levels of p19 and p40 promoter directed transcription increase, the Rep 52 and Rep 40 proteins alleviate the repression on the p5 promoter. Previous studies in our lab demonstrated complex formation between the Rep78/68 proteins and Rep52. In addition, the Rep78/68 proteins repress the p5 promoter. However, titration of increasing levels of Rep52 causes derepression of the p5 promoter. The present model supports a feedback mechanism in which the rising amounts of Rep52/40 act on the Rep78/68 proteins bound at the p5RBE. The Rep52 protein would cause an alteration of the large Reps on the p5RBE to eliminate repression of the p5 promoter (Fig. 5, C).

This feedback loop model between the products of the p5 and p19 promoters creates a steady-state level of transcripts. The molar transcript ratios of 1:10:100 for the

Fig. 5. **Model of Rep Feedback Regulation between the p5 and p19 Promoters.** *A*, Rep 78/68 bound to TR A-stem sequence transactivates the p5, p19, and p40 promoters. *B*, Increase of Rep protein concentration allows Rep 78/68 binding to p5 promoter repressing the p5 promoter. Rep bound at p5 promoter transactivates the p19 and p40 promoters. *C*, Higher p19 transcription creates more Rep52 protein which derepresses the p5 promoter.


A
E1A
E1A
Rep
YY1
YY1
p5
p19
p40
B
E1A
E1A
Rep
YY1
Rep
YY1
p5
p19
p40
C
E1A
Rep
E1A
Rep
YY1
Rep
YY1
p5
p19
p40

p5, p19, and p40 promoters maintain four relationships. First, the lower level of p5 transcription creates enough Rep78/68 to resolve the terminal repeats while preventing excess endonuclease and helicase activity from eliminating any transcriptionally competent AAV genomes. Second, the increasing Rep78/68 levels are autoregulated by their repression of the p5 promoter. Third, the synthesis of higher levels of Rep52/40 transcripts resulting from p5RBE trans-activation causes the derepression of the p5 promoter thereby increasing the Rep78/68 levels. Fourth, the p40 promoter is relatively unaffected by this feedback inhibition allowing constant transactivation of p40 and synthesis of the capsid transcripts.

CHAPTER 2
MATERIALS AND METHODS

Cell Lines and Virus.

Human A549 cells (ATCC #CCL 185) were maintained in Dulbeco's modified essential media (DMEM, Gibco BRL) containing 10% bovine calf serum (BCS, HyClone Laboratories Inc.) at 37°C in 100mm culture dishes. Adenovirus 5 was grown and titered on 293 cells.

Plasmids

The p5CAT plasmid is the same as the p5CAT190 plasmid described by Chang et al. (24) and was kindly provided by Tom Shenk. It contains AAV2 nucleotides 190-310 followed by the CAT expression cassette.

The pIM45 plasmid contains the AAV-2 nucleotides 145-4373 in a pBS M13+ vector (Stratagene Corporation). It essentially contains all of the AAV genome except for the terminal repeats.

pAXX is a modified pIM45 plasmid in which stop codons have been inserted by site specific mutagenesis at Rep78 amino acid 71 and at Rep52 amino acid 14. Thus, pAXX is incapable of producing either p5 or p19 Rep proteins, and it was used for the construction of all of the CAT containing plasmids below. pAXX, pIM45 and all other plasmids used in this study were sequenced to insure that the AAV sequences were correct and to confirm the positions of the mutations.

The p19CAT3 plasmid was created by inserting synthetic oligonucleotides within the pCAT3BASIC plasmid (Promega Corporation) that together reconstructed the

essential elements of the p19 promoter. The p19 synthetic sequence was created with three pairs of oligonucleotides that were annealed and then ligated together. The first oligonucleotide pair (cAAP-140) had the sequence 5'-CGTCACAAAGACCAGAAATGGCGCCGTCTCGAGTGACAATT-3' that contains AAV nucleotides 722-756 with a mutation that destroys the SP1-130 binding site and converts it into an XhoI restriction site. The second pair of oligonucleotides (p19XbaSac) had the sequence 5'-CTAGATCCGATGAGTGCTACATCCCCAATTACTTGCTCCCCAAAACCCAGCCT GAGCT-3' that contains AAV nucleotides 757-814 with the GGT-110 binding site converted into an XbaI restriction site. The third pair of oligonucleotides (SP1-50CAT), had the sequence 5'-CCAGTGGGCGTGGACCCGCGGGGAACAGTATTTAAGCGCCTA-3' that contained AAV nucleotides 815-869 with the TATA-35 site mutated into a SacII restriction site. The Sp1-130, GGT-110 and TATA-35 sites had been shown previously to have little effect on p19 transcription. All three oligonucleotide pairs were heated to 90°C and annealed together by slowly cooling to room temperature. They were then ligated using T4 DNA ligase (Life Technologies). After ligation, a small amount of the DNA product was used as template in a PCR reaction performed with two primers, p19XmaI 5'-ATACCCGGGCACAAAGACCAGAAATGGCG-3' and p19BglII 5'-ATAAGATCTCGTGAGATTCAAACAGGCGCTT-3'. The PCR reaction amplified the synthetic p19 promoter, which was digested with XmaI and BglII and cloned into pCAT3BASIC (Promega Corp.) vector that had been digested with the same restriction enzymes.

The p19CAT3-700 plasmid was created by subcloning an oligonucleotide containing the wild type p5 promoter Rep binding element (p5RBE) sequence, 5'-AAGCCCGAGTGAGCACGCAGGGTCTAGTACT-3', into the BsaAI restriction site of the p19CAT3 plasmid 700 nucleotides upstream of the start of transcription of p19. The p19CAT3-145 plasmid was created by subcloning the wild type p5RBE sequence into the MluI site at the –145 site of p19CAT3. The p19CAT3-100 plasmid was created by subcloning the wild type p5RBE sequence into the XhoI site at the –100 site of p19CAT3. The p19CAT3+25 plasmid was created by subcloning the p5RBE sequence into the BglII site at the +25 nucleotide site of p19CAT3. The p19CAT3+1225 plasmid was created by subcloning the p5RBE sequence into the +1225 site of p19CAT3.

The pIM45CAT3 plasmid was constructed by first digesting pAXX with BclI, which cleaves at AAV nucleotide 965. The linear pAXX DNA was ligated to the BamHI and BclI fragment of pcDNA3.1(+)CAT (Invitrogen Corporation) which contains the CAT gene. pIM45 contains AAV nucleotides 145-965 (including all of the p5 and p19 promoters) followed by the CAT gene. The psub262CAT3 plasmid was created in the same way by ligation of the BamHI and BclI fragment from pcDNA3.1(+)CAT into the psub262 plasmid, which contains a mutant p5 RBE.

The pIM45CAT3poly A plasmid was created in two steps. The SmaI to NruI fragment from pAXX containing the AAV p5 promoter as well as a portion of the Rep gene (AAV bp 145- 658) was inserted into the BsaA1 site of p19CAT3; this plasmid was called p5_p19CAT3. Then, the SmaI and SfiI fragment from the p5_p19CAT3 plasmid (containing AAV bp 145-544 and the late polyadenylation signal of SV40) was substituted for the NruI to SfiI fragment (containing AAV nucleotides 544-658) in the pIM45CAT vector. The resulting pIM45CAT3poly A contained AAV nucleotides 145-

544, followed by the SV40 late poly A site (563 bp), followed by AAV nucleotides 722-869 from p19CAT3 containing substitutions at Sp1-120, GGT-110 and TATA-20, followed by the CAT gene.

The 2xGAL4 plasmid was created by site-directed mutagenesis of the p5RBE and the p19 SP1-50 sites of pIM45CAT3 to GAL4 DNA binding sites. The p5RBE within pIM45CAT3 was mutated to a GAL4 DNA binding element using the oligonucleotide sequence 5'-GCGACACCATGTGGTCACGCTGGGCCGCGGCGGAAGACTCTCCTCCGAGGGTCTCC-3'. The p19 promoter SP1-50 site within pIM45CAT3 was mutated to a GAL4 DNA binding site using the oligonucleotide sequence 5'-CCCCAATTACTTGCTCCCCAAAACTCAGCCTGCGGAAGACTCTCCTCCGACTAATATGG-3'.

The pM-53 plasmid expressed a fusion protein of the GAL4 DNA binding domain and the mouse p53 protein containing p53 amino acids 72 to 390. It was supplied in the Mammalian MATCHMAKER Two-Hybrid Assay Kit (Clontech Laboratories). The pM-Tg plasmid contains the GAL4 DNA binding domain fused to SV40 T antigen amino acids 87-708. The SV40 T-antigen DNA fragment containing amino acids 87 to 708 was removed from the pVP16-T plasmid (Clontech Laboratories) by EcoRI and SalI digestion and inserted by directional cloning into EcoRI and SalI digested pM plasmid (Clontech Laboratories).

The 2xGAL4 subMLTF plasmid was created by site-directed mutagenesis of pIM45CAT3 2xGAL4 using the oligonucleotide 5'-GGTCTAGAGGTCCTGTATTAGATACACGCGTGTCGTTTTGCGACATTTTGCGACACC-3' that resulted in the replacement of the p5RBE site with the MluI restriction

site. The 2xGAL4 subYY1-60 plasmid was mutated at the YY1-60 binding site within the p5 promoter to EcoRI and BamHI sites using the oligonucleotide 5'-CCTGTATTAGATACACGCGTGTCGTTGAATTCTGGGATCCGACACCATGTGGT CACGC-3'. The 2xGAL4 subMLTF, YY1-60 plasmid was created by the site-directed mutagenesis of the 2xGAL4 subMLTF plasmid using the subYY1-60 oligonucleotide sequence 5'-CCTGTATTAGATACACGCGTGTCGTTGAATTCTGGGATCCGACACCATGTGGT CACGC-3'. The 2xGAL4 subMLTF, YY1-60, YY1+1 plasmid was created by site-directed mutagenesis of the 2xGAL4 subMLTF, YY1-60 plasmid using the oligonucleotide sequence of 5'-CGGCGGAAGACTCTCCTCCGAGGGTCTAAATTTTGAAG-3'.

Transfections

Plasmid transfections were performed using cationic liposomes (LipofectAMINE Plus, Life Technologies). Twenty-four hours before transfection, A549 cells were plated in 60mm dishes so the cells were 75% to 90% confluent on the day of transfection. The A549 cell media was removed and 2 ml of serum-free DMEM was added for the transfection procedure. Plasmid DNA was mixed with 250 µl of serum free DMEM containing 8 µl Plus Reagent (Life Technologies) and incubated at room temperature for 15 minutes. The plasmid DNA and Plus Reagent mixture was mixed with 250 µl of serum-free DMEM containing 12 µl of LipofectAMINE and incubated for another 15 min. The entire 500 µl of plasmid DNA/LipofectAMINE mixture then was incubated with the A549 cells without serum for three hours. At three hours post-transfection, 2.5 ml of DMEM with 20% bovine serum with or without Adenovirus 5 at a multiplicity of infection (MOI) of 5 pfu/cell was added to the A549 cells. To control for transfection

efficiency, each transfection contained 1 μg of pcDNA 3.1(+)gfp and 1.5 μg of pcDNA3.1(+)β gal. Extracts were then assayed for β gal activity using ONPG (Promega) to normalize for the transfection efficiency. In those cases where a linear CAT reporter plasmid was used, a linear β gal plasmid was also used.

CAT Reporter Assays

Forty-eight hours post-transfection, the A549 cells were washed with 2 mL PBS and lysed in 400 μl of lysis buffer (CAT Reporter Lysis Buffer, Promega Corporation). The whole cell extracts were vortexed for twenty seconds and heated at 65°C for 10 minutes to inactivate endogenous deacetylases. After heat inactivation, the extracts were centrifuged for 2 minutes and the clarified supernatant was transferred to a new tube. CAT activity was measured by a standard chloramphenicol acetylation reaction, which contained in 125 μl: 1x lysis buffer (Promega Corp.), 0.2 mM n-Butyryl Coenzyme A (Promega), 10 μM ^{14}C-chloramphenicol (Amersham Pharmacia Biotech, 58 mCi/mmol) and either 4 μg of protein from an adenovirus-infected extract or 20 μg of protein from an uninfected extract. The reaction was incubated for 24 hr at 37°C and then extracted with 300 μl of mixed xylenes (Fisher Scientific). The entire upper organic phase containing the acetylated chloramphenicol was back extracted two times with 100 μl of 0.25M Tris-HCl pH 8. The amount of acetylated chloramphenicol in 200 μl of the mixed xylenes phase was determined by liquid scintillation counting. Each construct was tested by transfection and CAT assay a minimum of three times. The average CAT activity (as cpm of acetylated product) was calculated after correction for the amount of extract used, and is reported +/- one standard deviation.

CHAPTER 3
RESULTS

Analysis of Adeno-Associated Virus Transcription

The overall goal of my research was to understand the regulation of the Adeno-Associated Virus promoters. In order to perform this analysis, the different AAV promoter elements were to placed upstream of reporter gene constructs which gauged the relative influence of the DNA sequence upon gene expression. In the previous studies of AAV transcriptional regulation, the analysis was performed by isolating a single AAV promoter region and mutating a series of single transcription factor binding sites (123). Another study focused on the effects of a single transcription factor upon all three AAV promoters was performed in the global genomic context of the AAV genome (143). In the context of the AAV genome, redundant transcription factor effects would not be detected through the loss of the single transcription factor. To address the combinatorial effects of multiple regulating factors on the AAV genome, my research goals focused on the determination of the non-structural AAV protein Rep as a transcriptional activator, defining the activity of Rep in AAV gene expression, and the role of a Rep-Sp1 interaction in the direct mechanism of AAV transcriptional regulation.

Define Rep Binding Element as Enhancer or Proximal Promoter Element

AAV must convert quickly from a latent virus, presumably integrated in chromosome 19 (93, 160), to a productive, replicating genome following Ad infection (133). To accomplish this, the virus appears to use two factors as a switch. The first is

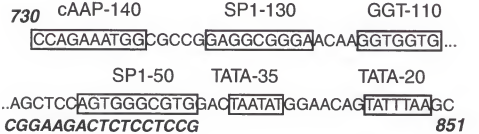
Rep protein, which in addition to YY1 and MLTF, binds upstream of the p5 promoter (Fig. 6) to shut off p5 transcription during latency (23, 97, 168). Thus, in the absence of Ad, Rep78 and 68 cooperate with cellular proteins to completely inhibit p5 transcription (143, 203). The second is adenovirus infection. Ad infection leads to E1a and Ad DBP mediated activation of p5 (23, 24, 102), which accomplishes two things. It increases transcription from the p5 and p19 promoters to produce increased levels of the four Rep proteins (9, 23, 102). Rep78 and 68, in turn, increase transcription from the p19 and p40 promoters to an even higher level (100, 123, 143, 203), while reducing transcription from the p5 promoter (143, 203). Ad infection also independently activates p19 transcription (9). Thus the p5 and p19 promoters are repressed by Rep in the absence of Ad and differentially regulated in the presence of helper virus. During the proviral stage, Rep autorepresses its synthesis to prevent excision of the provirus and expression of its gene products. In the presence of the helper virus, Rep induces the synthesis of the p19 proteins that required are for encapsidation and maintains the p5 Rep proteins at a lower level to optimize the level of replicating intermediates.

The p5 promoter (Fig. 6) has been shown previously to contain three elements involved in p5 regulation, an MLTF site at –80, a YY1 site at –60, and an RBE at –20 (23, 97, 125, 143, 168). In the absence of Ad infection, specifically E1a gene expression, all three factors (YY1, MLTF and Rep) repress p5 transcription (24, 74, 97, 98, 143). In the presence of an adenovirus coinfection, YY1 and MLTF both activate p5 transcription (24). In the case of YY1, this apparently occurs via an interaction of YY1 and E1a with the cellular p300 protein (103). Rep, however, continues to repress p5 when bound to the p5 RBE, but is now capable of activating the two downstream promoters, p19 and

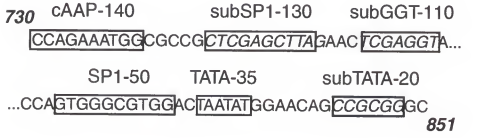
Fig. 6. **Sequences of the p5 and p19 promoters**. The known transcription elements in the p5 and p19 promoters are diagrammed along with their approximate positions upstream of the mRNA start sites. p5CAT plasmid contains only the p5 promoter elements driving CAT expression, while pIM45CAT3 contains both the p5 and p19 promoter elements with the p19 promoter driving CAT expression. Dotted lines indicate intervening sequences that are not shown. Shown in italics are substitutions within the p19CAT3 promoter at -120, -110, and -20 that were previously demonstrated to have little effect on p19 transcription and the sub262 substitution within the p5 RBE that no longer binds Rep protein. Shown in bold italics are the GAL4 substitutions in 2xGAL4 that replace the p5 RBE and the p19 SP1-50 sites. A CREB/ATF site, which has been mapped upstream of the MLTF site in p5, is not shown.

p5

p19



p19CAT3



p40 (98, 143, 203). In addition to the YY1, RBE and MLTF sites, p5 contains a conventional TATA site (-30) and a YY1 responsive initiator site (+1) (164, 178). In the presence of Rep and Ad gene expression, only mutations in the TATA, YY1-60 and MLTF sites significantly affect p5 transcription (143).

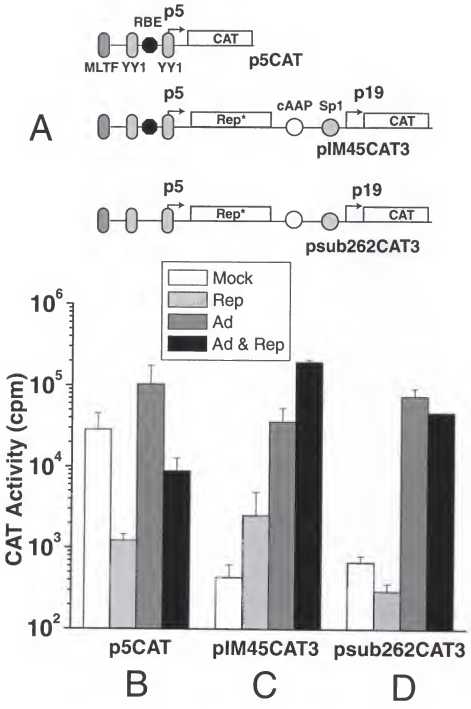
The p19 promoter (Fig. 6) contains two TATA sites (-30 and -35), which are redundant, two Sp1 sites (-50 and -130) and a site at -140 that binds an unidentified cellular AAV activating protein (cAAP) (27, 145, 178). Transactivation of the p19 promoter by Rep appears to require both the -50 Sp1 site and the -140 cAAP site (145). It also requires the p5 RBE (143). In the absence of the p5 RBE, the TR, presumably via its RBE, can transactivate all three promoters (9, 123, 143, 145, 203). Evidence that the RBE within the TR is probably responsible for transactivation comes from deletion analyses of the TR (9, 203). The two RBE elements appear to be redundant with respect to p19 transactivation at low Adenovirus MOIs but the TR predominates at high Ad MOI (>10) (9, 143, 203). Furthermore, at high multiplicities of infection of Adenovirus (>25), the effect of Rep78 or 68 expression on p19 activity is no longer seen; that is, expression of Ad factors can substitute for Rep78 or 68 (203). Finally, it has been shown that Rep and Sp1 can directly interact (68, 145), and that Rep bound to the p5 RBE can form a DNA loop with Sp1 at p19 (144).

Repression by Rep proteins is more complex. In contrast to transactivation, which appears to require either Rep78 or 68 plus Ad infection, repression of the p5 and p19 promoters occurs in the absence of Ad regardless of which Rep protein is expressed (74, 97). Repression of the p5 promoter depends in part on Rep binding to the p5 RBE but also requires a functional Rep ATPase (97, 98). However, repression of p5 can be

seen in the absence of a functional p5 RBE. Furthermore, the p19 promoter, which does not have an efficient RBE binding site, can be repressed by all four Rep proteins, including Rep52 and 40, which lack an RBE binding domain and contain only the Rep helicase and ATPase domains (74, 77, 97, 174).

Our initial hypothesis was that the upstream p5 RBE-Rep complex might behave like an enhancer sequence that activates transcription from downstream promoters. To determine if this were true we made a series of p19 CAT constructs (Figs. 7 and 8) in which CAT gene expression was driven by the p19 promoter. The first set of constructs (Fig. 7) was made to be certain that the CAT constructs would accurately reflect the control of p5 and p19 transcription that is seen with the parental TR minus pIM45 plasmid or with wild type AAV. In this set of constructs (Fig. 7), all of the sequences upstream of the p19 mRNA start site were identical to those of wild type AAV (pIM45CAT3) or contained a mutation in the p5 RBE (psub262CAT3) that eliminated Rep binding (125, 143). To prevent expression of aberrant Rep proteins from pIM45CAT3 and psub262CAT3, both plasmids contained an amber mutation at amino acid 71 of Rep78. This mutation terminated the synthesis of Rep78 while the CAT insertion prevented synthesis of Rep52, so that no Rep proteins could be detected in the absence of a Rep expressing plasmid. With the exception of the terminal repeat, these constructs contain all the sequences necessary for p19 regulation and p19 transactivation by Rep (123, 143, 145). The p5CAT plasmid described by Chang et al. (24) was used to monitor expression from the p5 promoter (Fig. 7). It contained all of the sequences upstream of the p5 promoter that have been shown to be necessary for p5 regulation with the exception of the TRs. All of the plasmids used in this study were missing the AAV

Fig. 7. **Activity of the wild type p5 and p19 promoters**. *A*, Diagram of the p5 and p19 promoter elements in the CAT reporter constructs p5CAT, pIM45CAT3 and psub262CAT3. The asterisk indicates a stop codon in the Rep78 open reading frame at amino acid position 71. *B-D*, Graphic representation of CAT activity from the p5CAT, pIM45CAT3, and psub262CAT3 reporter constructs after transfection in A549 cells in the presence of plasmid alone (Mock), or with the addition of 1 mg of pIM45 (Rep), Adenovirus at an MOI=5 (Ad), or 1 mg of pIM45 and Adenovirus at an MOI=5 (Ad & Rep).


RBE
p5
CAT
MLTF YY1 YY1
p5CAT
A
p5
Rep*
cAAP Sp1
p19
CAT
pIM45CAT3
p5
Rep*
p19
CAT
psub262CAT3
Mock
Rep
Ad
Ad & Rep
CAT Activity (cpm)
10^6
10^5
10^4
10^3
10^2
p5CAT
pIM45CAT3
psub262CAT3
B
C
D

TRs because we and others have shown that the TRs are redundant activation elements that promote excision and replication, thereby changing the template copy number available for transcription (9, 123, 143, 159, 203).

The three plasmids (p5CAT, pIM45CAT3 and psub262CAT3) were transfected into A549 cells in the presence or absence of a coinfection with Adenovirus (MOI=5) or in the presence or absence of a cotransfection with the wild type Rep expressing plasmid pIM45 (Fig. 7). pIM45 was chosen because we and others have shown that the four Rep proteins Rep78, 68, 52, and 40 are expressed at different levels and have differential effects on repression and transactivation of p19 during productive infection (74, 97, 143), and because pIM45 expresses the Rep proteins at approximately the same relative levels that are seen during wild type viral infection (123, 203). Cells were harvested approximately 40 hours after plasmid transfection and Ad infection and cell free extracts were assayed for CAT activity as described in Methods.

As expected, coinfection with Ad alone significantly induced p5 transcription, approximately 4 fold (Fig. 7 B). This presumably occurs through the interaction of the E1a proteins of Ad with the p300-YY1 complex bound to the p5 promoter (24, 103, 168). Cotransfection with a Rep expressing plasmid repressed p5 transcription both in the absence (20 fold) and in the presence (12 fold) of Ad coinfection. This has also been observed previously (9, 74, 98, 143) and is due in part to binding of Rep to the p5 RBE. In contrast, p19 transcription was significantly induced by Rep expression both in the presence and the absence of Ad coinfection, about 5-6 fold (Fig. 7 C). This induction did not occur if the p5 RBE was mutated so that it could no longer bind the Rep protein (Fig. 7 D). Ad coinfection alone also significantly induced p19 transcription, approximately

80 fold, and this occurred to approximately the same level regardless of whether the p5 RBE element was intact (Fig. 7, C and D). The induction by Ad and Rep appeared to be multiplicative and together they induced transcription from the p19 promoter by almost 450 fold (Fig. 7 C). p19 CAT activity in the presence of Rep and Ad was approximately 20 fold higher than p5 CAT activity. This 20 fold difference was approximately the same as that seen late after infection during normal wild type AAV infections when transcripts are compared by other methods such as Northerns (9, 100). Thus, the CAT constructs used in this study seemed to accurately reflect mRNA steady state levels seen during wild type AAV infections or those seen with TR minus plasmid transfections.

Finally, we noted that when basal expression from the psub262CAT3 plasmid was compared with that seen in the presence of the Rep expressing plasmid, there was approximately a 2 fold inhibition of p19 transcription (Fig. 7 D). Similarly, when the level of expression in the presence of Ad was compared with expression in the presence of Ad plus Rep, there was approximately a 2 fold inhibition of p19 transcription by Rep protein. Thus, expression of the Rep protein caused inhibition of the p19 promoter in the absence of the p5 RBE. This has also been reported previously (9, 97).

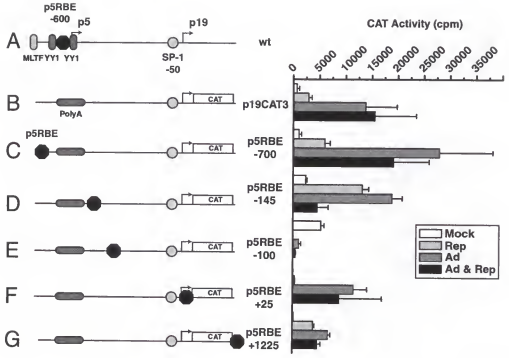
The p5 RBE is not an upstream activation signal.

Having confirmed that the p5 RBE was essential for Rep mediated induction of p5 transcription, we then asked whether the p5 RBE behaved like a position independent enhancer or like an upstream activation signal. If the p5 RBE behaved like a conventional enhancer or upstream activation sequence, we expected that an artificial construct containing all of the essential p19 proximal promoter elements would continue to respond to an upstream RBE in the presence of Ad and Rep gene expression. For this purpose we designed a series of CAT constructs in which the p19 proximal promoter

elements were left intact but the upstream AAV sequences, including the p5 promoter were substituted with bacterial DNA. In these constructs the p19 sequences up to -150 bp of the mRNA start site were essentially the same as the wild type sequence (present in pIM45CAT3) except that the TATA site at -35, the GGT site at -110 and the Sp1 site at -130 were mutated (Fig. 6). These sites had been shown previously to be non-essential for Rep induction or basal p19 activity (145). The CAT coding sequence was inserted at +92 of the p19 message and a poly A signal was inserted upstream of the p19 promoter to prevent read through from fortuitous upstream polymerase II promoters in the bacterial sequences. The p5 RBE signal was then inserted at various positions upstream or downstream of the p19 promoter. In the control plasmid, p19CAT3, the p5 RBE was omitted.

The level of p19 activation seen with the p19CAT3 plasmid in the presence of either Rep or Ad alone was similar to that seen with the parental pIM45CAT3 plasmid that contained all of the upstream AAV sequences including the p5 promoter elements. As expected, Rep did not significantly induce p19 when Rep and Ad were present together if there was no upstream RBE (Fig. 8 B). Surprisingly, Rep also did not induce p19 transcription in the presence of both Rep and Ad when an RBE was inserted at -700 upstream of the p19 promoter. This is approximately the same position that the p5 RBE occupies in the parental plasmid pIM45CAT3. Furthermore, Rep did not activate p19 transcription (compare Ad only with Ad plus Rep lanes) regardless of where the RBE was inserted upstream or downstream of the p19 mRNA start site. Instead, the RBE acted like a repressor element at most of the positions tested in the presence of both Rep and Ad, and the level of repression increased dramatically as the RBE was inserted closer

Fig. 8. **Effect of RBE position on p19 activity**. *A*, Diagram of transcription elements within the wild type p5 and p19 promoters. *B-G*, Diagrams of the parental plasmid p19CAT3 (*B*) and derivatives of p19CAT3 C-G, in which an RBE was inserted at various positions with respect to the start of p19 transcription (-700 to +1225). The p19CAT3 plasmid has the minimum p19 promoter elements as shown in Fig. 6 and no p5 promoter elements. The CAT activity of each construct was determined after transfection into A549 cells in the presence of plasmid alone (Mock), pIM45 (Rep), Adenovirus (Ad) or pIM45 and Ad (Ad & Rep).


p5RBE
-600
p5
p19
A
wt
MLTF YY1 YY1
SP-1
-50
CAT Activity (cpm)
0
5000
10000
15000
20000
25000
30000
35000
B
CAT
PolyA
p19CAT3
p5RBE
C
CAT
p5RBE
-700
D
CAT
p5RBE
-145
E
CAT
p5RBE
-100
F
CAT
p5RBE
+25
G
CAT
p5RBE
+1225
Mock
Rep
Ad
Ad & Rep

to the p19 promoter. The most dramatic repression was seen when the RBE was placed at -100 upstream of the p19 start site (compare Fig. 8 B, Ad + Rep with 8 E, Ad + Rep). When the RBE was placed downstream of the p19 start site (Fig. 8 F and G), some of the p19 activity was recovered but the RBE continued to generally repress p19 transcription even when it was placed far downstream at +1225. Curiously, in the presence of only Rep gene expression, p19 activity was significantly induced over basal levels by Rep when the RBE was placed at -700 or -145.

We concluded from these experiments that the RBE was not a conventional upstream activation signal or an enhancer. It did appear, however, to have the ability to act as a general repression signal in the presence of adenovirus infection under almost all conditions tested when it was closer than 700 bp upstream or 1200 bp downstream of the p19 promoter. Our data also suggested that Rep itself was probably not an activator and left open the question of how the p5 RBE could transactivate p19 in the context of the normal AAV sequence.

Activation of p19 is not due to read through from p5.

We considered two other possible mechanisms of p19 activation by the p5 RBE. One was that transcription from the p5 promoter activated p19 by a read through mechanism. Although poorly understood, this has been shown to occur in the case of the adenovirus E1b gene. In this case, active transcription of the E1a promoter causes read through activation of the E1b promoter in a cis-dependent manner (45, 121). E1a transcription appears to alter the adenovirus genome into a transcriptionally competent template before Ad DNA replication and late transcription have begun. This possibility was not likely in the case of the p5/p19 promoters because the p5 RBE inhibited CAT expression (Fig. 7) from the p5 promoter at the same time that it induced CAT expression

from the p19 promoter. However, it remained possible that CAT expression in this study (as well as other steady state mRNA measurements done previously) did not reflect the true firing rate of the p5 promoter. In order to address the possibility that p5 transcription may be transactivating p19, two kinds of experiments were done. In the first experiment, we compared p19 CAT activity from the pIM45CAT3 plasmid in the presence of Ad or Ad plus Rep when the physical connection between the p5 and p19 promoters was removed by a restriction digest (Fig. 9). The pIM45CAT3 plasmid was transfected either uncut, cut with Sph1, which cuts outside both the p5 and p19 promoters, or cut with Nru1, which cuts between the p5 and p19 promoters. As before, the supercoiled uncut plasmid showed strong activation of the p19 promoter by Rep in the presence of Ad. This was not seen when the plasmid was linearized by restriction digestion at either restriction site even after the input DNA levels were normalized (see Methods). Thus, Rep induction appeared to require a superhelical template. The difference in CAT expression between the Sph1 and Nru1 cut plasmids in the presence of adenovirus alone or Ad plus Rep was 2-3 fold. Thus, read through activation was not likely to contribute more than 2-3 fold to p19 activity.

In the second experiment, we inserted a poly A site between the p5 and p19 promoters of pIM45CAT3 and psub262CAT3. Insertion of a poly A site between p5 and p19 in the psub262CAT3 plasmid had a minimal (2-3 fold) effect on p19 activity (Fig. 10 D and E). This suggested again that relatively little if any p19 transcription relied on read through from the upstream p5 promoter. In contrast, when the p5 RBE was present (Fig. 10 B and C), insertion of the poly A site decreased p19 activity by 10-20 fold under all conditions tested. Regardless of whether a poly A site was inserted, the plasmids that

Fig. 9. **Linearization of pIM45CAT3 plasmid to inhibit read through transactivation**. p19 CAT activity from A549 cells transfected with supercoiled pIM45CAT3 plasmid. *A*, or plasmid that had been linearized by digestion with NruI (*B)* or SphI (*C)*. For each case the activity in the presence of Adenovirus infection, MOI=5 (Ad) is compared with the activity in the presence Ad infection and transfection with 1 mg of pIM45 (Ad & Rep). The values in (*A*) are the same as those in Fig. 7C and are presented for comparison.

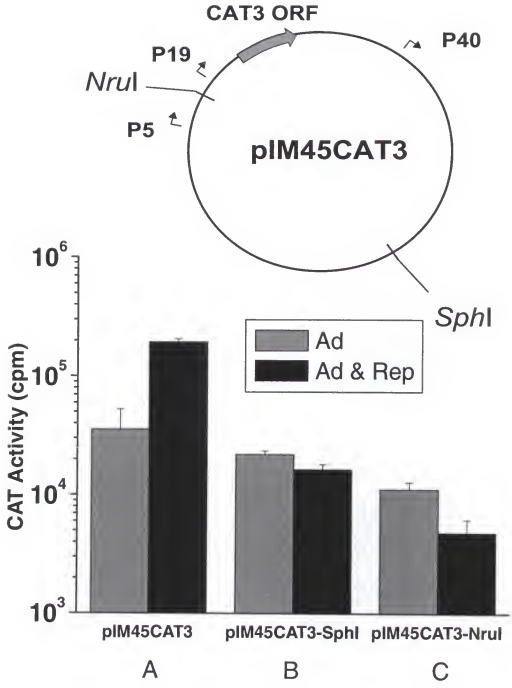
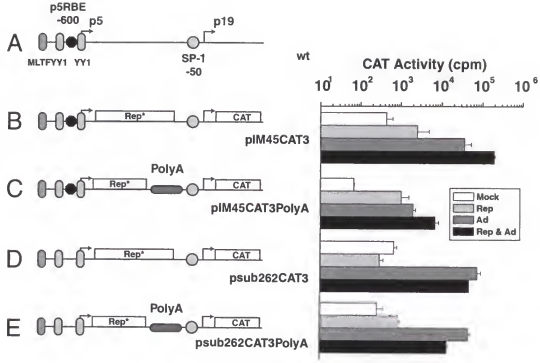

CAT3 ORF
P19
P40
NruI
P5
pIM45CAT3
SphI
Ad
Ad & Rep
CAT Activity (cpm)
10^6
10^5
10^4
10^3
pIM45CAT3
pIM45CAT3-SphI
pIM45CAT3-NruI
A
B
C

Fig. 10. **Effect of poly A insertion to inhibit read though activation**. *A,* Poly A sequence was inserted into pIM45CAT3 and psub262CAT3 between the p5 and p19 promoters to determine the effect of a poly A signal on p19 activity when the plasmid was transfected by itself (Mock), in the presence of the Rep expressing plasmid pIM45 (Rep), in the presence of Ad at an MOI of 5 (Ad), or in the presence of both pIM45 and Ad (Rep & Ad). The asterisks indicate that the Rep coding sequence has a stop codon at Rep78 amino acid position 71 to prevent Rep expression from the CAT reporter plasmids. The values in (*B*) and (*C*) are the same as those in Fig. 7 *C* and *D* and are presented here for comparison.


p5RBE
-600
p5
p19
A
MLTFYY1
YY1
SP-1
-50
wt
CAT Activity (cpm)
10^1
10^2
10^3
10^4
10^5
10^6
B
Rep*
CAT
pIM45CAT3
PolyA
C
pIM45CAT3PolyA
Mock
Rep
Ad
Rep & Ad
D
psub262CAT3
E
psub262CAT3PolyA

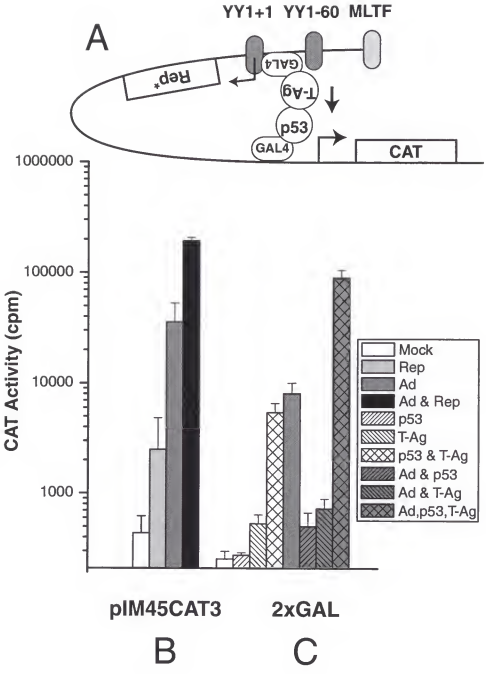
contained a p5 RBE both showed a Rep mediated induction of p19 in the presence of Ad. Taken together, our results suggested that modification of the p19 plasmid template by a method that could change the secondary structure of the plasmid (restriction digestion) or potentially create new protein complexes in the region between p5 and p19 (insertion of a poly A site) reduced p19 activity. However, they did not suggest read through activation as a likely mechanism for p19 induction.

Transactivation of p19 can be modeled in the absence of Rep.

Thus far, our data were not consistent with the p5 RBE being a conventional upstream activation signal or with the possibility that it stimulated p19 transcription by read through from the p5 promoter. If Rep bound to the p5 RBE was not itself a transactivator, we then considered the possibility that it had an architectural role. We had shown previously that Rep bound to the p5 RBE could form a DNA loop via protein interaction with Sp1 bound to the p19 promoter (144). It was possible that the interaction between Rep and Sp1 served primarily to bring the other p5 transcriptional elements (namely the MLTF and YY1 complexes) to the p19 promoter, and that one of these elements was primarily responsible for p19 activation. If this were true, then it should be possible to substitute the Rep and Sp1 sites with alternative elements that bound proteins that were capable of interacting.

To test this possibility we substituted both the p5 RBE and the p19 -50 Sp1 site within the parental plasmid pIM45CAT3 with Gal4 binding elements to make the plasmid called 2xGAL (Fig. 11). Substitution of the RBE and Sp1 sites was done so that the spacing in the p5 and p19 promoters remained the same (Fig. 6). We then tested for p19 activation in the presence of two Gal4 fusion proteins that contained the well characterized interaction domains of SV40 T antigen and p53 (47, 109) but did not

Fig. 11. **Replacement of Rep & Sp1 with Hybrid GAL4 Factors**. *A*, The diagram illustrates that the RBE in the p5 promoter and the Sp1-50 site in the p19 promoter of pIM45CAT were both replaced with GAL4 binding sites. See Fig. 6 for specific sequence modifications. In the presence of hybrid GAL4-p53 and GAL4-Tag fusion proteins, the remaining p5 promoter elements and their associated protein complexes would be brought into close proximity with the p19 promoter resulting in a DNA loop structure. *B*, Transactivation of the pIM45CAT3 reporter plasmid when transfected alone (Mock), with 1mg of pIM45 plasmid (Rep), with an adenovirus infection, MOI=5 (Ad), or an Adenovirus infection at MOI=5 + pIM45 transfection (Ad & Rep). (This data is identical to that in Fig. 7 B and is presented here for comparison.) *C*, Transactivation of the 2xGAL plasmid when transfected alone (Mock), with 1 mg of pM-53 plasmid (p53), with 1 mg of pM-Tg plasmid (T-Ag), with 1 mg of both pM-53 and pM-Tg plasmid (p53 & T-Ag), with Ad infection at MOI=5 (Ad), with Ad infection at MOI=5 and the two hybrid plasmids (Ad, p53, T-Ag) or with Ad at MOI=5 and either 1 mg pM-53 (Ad & p53) or 1 mg pM-Tg (Ad & T-Ag).


A
YY1+1
YY1-60
MLTF
Rep+
GAL4
T-Ag
p53
GAL4
CAT
1000000
100000
10000
1000
CAT Activity (cpm)
Mock
Rep
Ad
Ad & Rep
p53
T-Ag
p53 & T-Ag
Ad & p53
Ad & T-Ag
Ad,p53,T-Ag
pIM45CAT3
2xGAL
B
C

themselves contain transcriptional activation domains. Substitution of GAL4 binding sites for the p5RBE and p19 Sp1 –50bp binding sites within the pIM45CAT3 reporter plasmid was expected to allow the GAL4 DNA binding domains to bind DNA and create a DNA loop between the two promoters through the p53 and SV40 T-Antigen interaction domains (Fig. 11A).

As expected, basal p19 CAT activity from the 2xGAL plasmid was low and comparable to that seen with the parental pIM45CAT3 plasmid (Fig. 11, B and C, compare Mock lanes). Expression of the Gal4 DNA binding proteins alone induced p19 activity by approximately 20 fold while infection with Ad alone induced p19 activity by approximately 30 fold. However, when both the Gal4 fusion proteins and Ad were present, p19 activity was induced by 350 fold over the basal level. Transfection with only one of the GAL4 fusion plasmids (p53 or T-ag) and infection with Ad did not induce p19 activation. These results demonstrated that an interaction between an activated p5 promoter, which occurs in the presence of Ad infection, and the p19 promoter was essential for p19 activation, and that this could be engineered in the absence of Rep and Sp1 elements. It also suggested that Rep and Sp1 probably function primarily as architectural proteins and activate p19 by bringing other p5 elements into close proximity with the p19 promoter.

The level of p19 activity seen in the presence of the two Gal4 fusion proteins and Ad was approximately half of that seen in the parental pIM45CAT3 plasmid in the presence of Rep and Ad (compare Fig. 11B, Rep with 11C, Ad, p53, T-ag). This was consistent with the fact that unlike the pIM45CAT3 plasmid, which binds Rep and Sp1 at distinct sites, the 2xGAL plasmid could bind the same fusion protein (either T antigen or

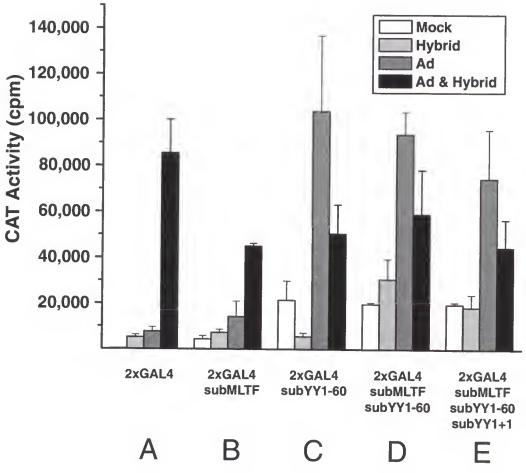
p53) at each GAL4 binding site (p5 and p19). When this happened it would lead to little or no interaction between p5 and p19, and presumably no activation. This would theoretically occur 50% of the time; thus, the 2xGAL plasmid was expected to be activated about half as well as the parental pIM45CAT3 plasmid. Indeed, transfection with only one of the GAL4 fusion proteins in the presence of Ad inhibited the activation normally seen with Ad (Fig. 11C, Ad & p53 or Ad & T-ag lanes). Alternatively, the Sp1-50 site may contribute to the basal level of the p19 promoter or to its ability to be induced by the Ad E1a gene product independently of an interaction with the p5 promoter elements.

Which p5 element is primarily responsible for Rep mediated induction of p19?

If the role of the p5 RBE is to bring some other element to the p19 promoter, which of the known p5 promoter elements (MLTF, TATA or YY1) is primarily responsible for Rep induced activation in the presence of adenovirus? To determine this we constructed a series of 2xGAL plasmids in which one or more of the other p5 transcription elements were mutated to eliminate binding of its cognate transcription factor (Fig. 6). Mutation of the p5 MLTF site had little effect (about 2 fold) on the final level of p19 activity (Fig. 12A and B). Furthermore, p19 activity was induced in the presence of Ad and both of the hybrid GAL4 fusion proteins, suggesting that MLTF was not critical for p19 induction (Fig. 12B). In contrast, any mutant that included a defective YY1-60 site was defective for induction in the presence of both Ad and the GAL4 factors (Fig. 12C, D, E). This suggested that at least one of the critical elements within the p5 promoter for p19 activation by a putative Rep-Sp1 interaction was the YY1-60 site.

Two other points became clear from an examination of the mutants. First, mutation of the YY1-60 site had a major effect on the basal activity of the p19 promoter.

Fig 12. **Transactivation of the 2xGAL p19 promoter by mutant p5 promoters**. The 2xGAL plasmid containing a wild type or mutant p5 promoter was transfected alone (Mock), with 1 mg each of the hybrid GAL4-p53 and GAL4-Tag expressing plasmids, pM-53 and pM-Tg (Hybrid), with an Adenovirus infection at MOI=5 (Ad), or with an Adenovirus infection at MOI=5 and the hybrid GAL4 expressing plasmids (Ad & Hybrid). See Fig. 6 for specific sequence modifications. *A*, Transactivation of the 2xGAL4 plasmid containing a wild type p5 promoter. (These values are extracted from Fig. 6B and presented here for comparison.) *B*, Transactivation of the 2xGAL reporter construct lacking the p5 promoter MLTF binding site. *C*, Transactivation of the 2xGAL reporter construct lacking the p5 promoter YY1 –60 binding site. *D*, Transactivation of the 2xGAL reporter construct lacking the p5 promoter MLTF and YY1 –60 binding sites. *E*, Transactivation of the 2xGAL reporter construct lacking the p5 promoter MLTF, YY1 –60, and YY1+1 binding sites.


CAT Activity (cpm)
140,000
120,000
100,000
80,000
60,000
40,000
20,000
Mock
Hybrid
Ad
Ad & Hybrid
2xGAL4
2xGAL4 subMLTF
2xGAL4 subYY1-60
2xGAL4 subMLTF subYY1-60
2xGAL4 subMLTF subYY1-60 subYY1+1
A
B
C
D
E

Mutation of this site in the p5 promoter increased basal transcription from the p19 promoter by 100 fold (compare Fig. 12A mock with 12C mock), and in the presence of Ad, p19 transcription was increased 10 fold (Fig. 12A, Ad and 12C, Ad). The YY1-60 element has been shown previously (24, 168) to repress the p5 promoter in the absence of Ad or Rep. Our data (Figs. 12A, C) indicates that the YY1-60 site also has a negative effect on p19 transcription both in the presence and the absence of Ad. In these two cases, no GAL4 fusion proteins were present; thus, interaction between the p5 and p19 elements through the Gal4 sites was not possible. This means that other kinds of interactions between p5 and p19 must be occurring in addition to those that occur between the Gal4 sites (ie., the p5 RBE and the p19 Sp1 sites). Second, mutation of the MLTF site also produced an increase (15 fold) in the basal level of p19 expression (Fig. 12A and B, mock lanes). Like the YY1 site, the MLTF site had been shown previously to repress p5 in the absence of Ad (24). Our results indicate that it also represses the p19 promoter in the absence of Ad, possibly by a set of p5/p19 interactions that are distinct from those mediated by the YY1 site and the Gal4 (Rep/Sp1) sites.

Transcriptional Analysis of Mutant Rep Proteins

Multiple studies of the AAV Rep proteins have demonstrated that the enzymatic activites of Rep could be localized into functional subunits within the protein. However, the complete elimination of one biological activity usually results in the loss of several others. The best example of this interactive effect is the nucleotide binding domain mutations. The loss of this ATPase domain removes Rep trs endonuclease activity and DNA replication but also creates a defect in transcriptional transactivation for both the p19 and p40 promoters (124). As result, a more detailed mutagenesis of the AAV Rep

protein was required to identify discrete protein domains for each individual enzymatic activity.

A previous study used the common method of charge-to-alanine mutagenesis for the production of temperature sensitive mutations. After an initial screening of 10 different Rep mutants, they isolated 2 mutants with separate conditional lethal phenotypes. The first mutant had an initial protein instability defect that was characterized as a Mg2+ binding mutation (50). This Mg dependent mutant was not required for Rep DNA binding or helicase but was critical for terminal repeat endonuclease activity. The second mutant was identified as a temperature sensitive mutation that was defective for intracellular replication of AAV. This temperature sensitivity created a 3-log titer difference between the permissive 32C temperature and nonpermissive 39C temperature.

Generation of Rep Mutant Plasmids.

In order to produce a large number of possible AAV Rep ts mutants, a charge-to-alanine amino acid mutagenesis approach was performed on the Rep open reading frame. This process is believed to disrupt functional domains on the protein surface and result in conditional lethal mutants. Within the plasmid backbone of pIM45, a noninfectious genomic subclone of the AAV-2 genome, 20 different clusters of charged amino acids were substituted with alanine amino acids (Table 1). The pIM45 plasmid allowed the resulting protein sequence changes to be expressed in all four Rep proteins. Mutagenesis in every Rep protein would allow possible conditional lethals to be assayed for in vivo biological effects. The expression of both Rep78/68 and Rep52/40 mutant proteins could be used to detect conditional lethals within assays for terminal repeat endonuclease activity, DNA replication, and viral transcription. Previous research and site-directed

Table 1. **Biochemical Activities of Rep Alanine Scanning Mutagenesis Panel.** In this Table, each Rep mutant was assayed for its ability to function as a possible mutant in Adeno-Associated Virus's known biochemical activities. Within the plasmid backbone of pIM45, a noninfectious genomic subclone of the AAV-2 genome, 21 different clusters of charged amino acids were substituted with alanine amino acids.

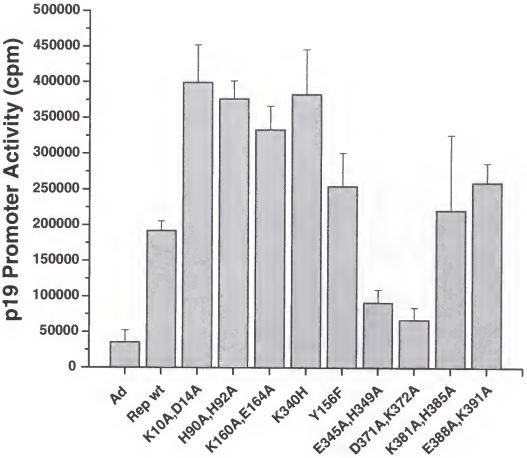
Amino Acid Change(s)	Titer	*in vivo* Replication	Binding	ATPase	m13 helicase	Nick Double-Stranded Template	Nick No-Stem Template
wild type	++	+	+	++	++	+	+
K10A,D14A	--	--	+	++	++	--	--
D40A,D42A,D44A	ts	ts	ts	++	++	ts	+
H90A,H92A	--	--	+	++	++	--	--
Y156F				+	++	--	--
K160A,E164A	--	--	+	++	++	--	--
K340H				--	--	--	-
E345A,H349A	--	--	+	+	+	--	-
D371A,K372A	--	--	+	+	++	--	+
K381A,H385A	--	--	n/d	n/d	n/d	n/d	n/d
E388A,K391A	--	--	+	+ / --	+ / --	--	+
E32A,K33A,E34A	++	+	n/d	n/d			
R68A,R69A,K72A	++	+	n/d	n/d			
D149A,E150A	++	+	n/d	n/d			
R223A	++		n/d	n/d			
D233A,K234A	++	+	n/d	n/d			
K278A,D282A	++	+	n/d	n/d			
E291A,D292A	++		n/d	n/d			
D455A,H456A,D457A	++		n/d	n/d			
E481A,E482A	++	+	n/d	n/d			
D501A,D504A	++	+	n/d	n/d			
D519A,E521A	+	+	n/d	n/d			

mutagenesis of the AAV Rep protein has resulted in the preliminary identification of a temperature sensitive mutant (50). This mutant D40A, D42A, D44A had a delayed replication phenotype that resulted in a 3 log difference in viral titer between 32C and 39C. We have determined the enzymatic activity affected by this protein sequence change as well as screened a panel of 20 new Rep charge-to-alanine mutants. We have assayed their ability to exhibit a temperature sensitive phenotype and localized the specific enzymatic activities affected within these replication defective mutants.

Transcriptional Activation of the p19 Promoter by Rep Mutants.

In order to directly examine the transcriptional activation of each charge-to-alanine mutant on the p19 promoter, pIM45CAT3 was used to assay each Rep mutant for its ability to specifically transactivate the p19 promoter during Adenovirus infection. The pIM45CAT3 construct had a strong level of Rep mediated transactivation that is comparable to previously published results (Fig. 13 Lanes 1 and 2) (Lackner and Muzyczka in press). However, the cotransfection of several replication defective mutants during an Adenovirus infection resulted in even higher levels of p19 transcriptional activation (Fig. 13 lanes 3,4,5,6, and 11). One of these mutants, K340H, is the well characterized nucleotide binding mutant (27, 38, 41, 74, 83, 90, 190, 195, 196, 203, 204). Previous studies have shown that this mutant is defective for both ATPase and M13 helicase activity but retains DNA binding activity. Weger et al. (203) have assayed its ability to transactivate the p19 and p40 promoters which resulted in much higher levels of AAV gene expression. Although this same K340H mutant was unable to stimulate gene expression in an AAV construct containing the terminal repeats, the loss of transcriptional activation was believed to be the result of the loss of DNA replication on the transcriptional template and not its inability to transactivate the p19 promoter.

Fig. 13. **Transcriptional Activation of the p19 Promoter by Rep Mutant Panel.** pIM45CAT3 reporter plasmid was used to assay each Rep mutant protein for its ability to transactivate the p19 promoter. A Rep protein expression plasmid, pIM45, containing the various alanine scanning mutants was co-transfected with Adenovirus to transactivate the p19 promoter.


p19 Promoter Activity (cpm)
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
Ad
Rep wt
K10A,D14A
H90A,H92A
K160A,E164A
K340H
Y156F
E345A,H349A
D371A,K372A
K381A,H385A
E388A,K391A

Another mutant construct, Y156F, was used as an experimental control in the pIM45CAT3 transcriptional assay. This mutant has specifically targeted the tyrosine residue at amino acid position 156, which has direct role in the endonuclease cleavage reaction during AAV DNA replication. The AAV terminal repeat is cleaved by the tyrosine residue and substitution of a phenylalanine at this position created a replication defective mutant (39, 173, 195). The Y156F mutant had no significant difference in its ability to transactivate the p19 promoter in the pIM45CAT3 plasmid background (Fig. 13 lane 7). This mutant has a functional ATPase and helicase activity (39, 173, 195.)

The three other mutants retain their ATPase and helicase activity but also express much higher levels of p19 gene expression (Fig. 13 lanes 3, 4, and 5). All three of these mutants are able to bind the terminal repeat but are unable to nick at the trs site. One reason for this higher expression could be an alteration in Rep-DNA binding on the p5 promoter. The formation of stable Rep protein complexes on the p5 RBE may allow the DNA loop between the p5 and p19 promoters to occur more often and transactivate the p19 promoter through Sp1 at a much higher rate. Another reason for a higher level of p19 transactivation may be a difference in binding affinity between the mutated Rep proteins and the Sp1 protein bound at the p19 promoter.

The most interesting result was the dramatic decrease in p19 promoter transactivation for the E345A, H349A and D371A, K372A mutants. These mutants have maintained their DNA binding, ATPase, and helicase activities although they are defective in p19 promoter transactivation. The p19 promoter assay using the pIM45CAT3 reporter gene plasmid was defective for both of these mutants as compared to the wt Rep protein. Although they were above the level of p19 transactivation with

Adenovirus alone, the mutants did not completely complement the ability of Rep to mediate p19 transactivation (Fig. 13 lanes 8 and 9). The disruption of Rep mediated transactivation in both of these mutants may be in different protein domain but may affect a similar overall function. The E345A, H349A mutant has an interesting characteristic in that it mutates a potential HMG box that may contribute to a possible mechanism of transactivation. The potential HMG box domain may be responsible for separation of DNA strands and the initiation of gene expression for this mutant Rep protein. The alteration of this protein domain may prevent the correct level of p19 transactivation through the action of Rep invading the DNA strands at the p5 or terminal repeat. The HMG box may permit the correct Rep protein complex to form on the p5 promoter and correctly contact the Sp1 bound at the p19 promoter. This requirement of strand invasion may be dependent on helicase activity as well as a functional HMG protein domain.

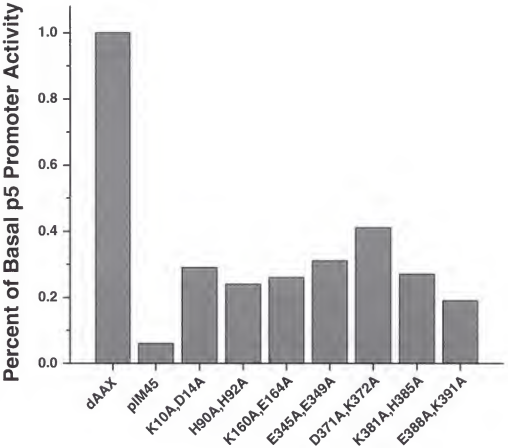
The D371A, K372A does not appear to directly affect any identifiable protein domains but its location is close to both a strong Rep-Rep interaction domain and a previously identified topoisomerase protein sequence. This protein domain could be responsible for the assembly of large Rep protein complexes that form on the various RBEs within the AAV genome. This mutant does not initially appear to target a critical domain required for Rep-Rep interactions but it appears to have multiple problems regulating the transcriptional activation of the p19 promoter as well as the ability of Rep to repress the p5 promoter.

Repression of the p5 promoter.

One of the most important characteristics of the AAV lifecycle is to repress the expression of the Rep gene in the absence of an Adenovirus infection (98, 143). In order to localize the Rep protein domain responsible for this biological activity, the entire Rep

mutant panel was assayed for its ability to repress p5 transcription in the absence of an Adenovirus coinfection. In this assay of the p5 promoter, the p5CAT3 promoter was transfected into A549 cells and assayed for its ability to be repressed in the presence of a mutant Rep protein. As a negative control, the nonsense mutant Rep, dAXX, was transfected to determine the basal strength of the p5-190CAT promoter (Fig 14 lane 1). When the wt Rep protein was transfected into A549 cells, the transcriptional activity of the p5 promoter is 6% of its basal activity demonstrating a strong level of repression by the Rep protein (Fig. 14 lane 2). It was interesting to note that all of the Rep replication mutants that were defective in transactivation of p19 were also greatly reduced in their ability to repress the p5 promoter. Strikingly, the two mutants E345A, H349A and D371A,K372A exhibited much lower Rep mediated transactivation of the p19 promoter and were unable to repress the p5 promoter through Rep-DNA binding activity. Each of these two mutants had 7-fold higher levels of p5 promoter expression resulting from the inability of these Rep proteins to correctly bind the p5 RBE and repressing the promoter (Fig 14 E345A, H349A and D371A,K372A). The modification of the HMG box may result in Rep protein which is unable to correctly assemble large Rep complexes and repress the p5 promoter. The highest degree of lower repression of the p5 promoter is directly related to its distance to the HMG box domain.

Fig. 14. **Transcriptional Repression of the p5 Promoter by Rep Mutant Panel**. p5CAT reporter plasmid was co-transfected with various Rep alanine scanning mutations. The basal level of p5 promoter activity was graphically shown as 1.0-fold of activity. The ability of each Rep mutant to repress the p5 promoter was shown as a percentage of p5 basal activity.


Percent of Basal p5 Promoter Activity
1.0
0.8
0.6
0.4
0.2
0.0
dAAX
pIM45
K10A,D14A
H90A,H92A
K160A,E164A
E345A,E349A
D371A,K372A
K381A,H385A
E388A,K391A

CHAPTER 4
DISCUSSION

To learn more about how the Rep protein controls AAV transcription, we tested several mechanisms of transcriptional control. Using the CAT gene as a reporter, we first confirmed that activation of the p19 promoter by Rep was dependent on a functional RBE within the p5 promoter (Fig. 7). As expected p19 activity was significantly reduced in the absence of Rep or in the absence of the p5 RBE, provided that the cells had been infected with adenovirus. This confirmed the results of several previous studies including our own (100, 123, 143, 203). In addition, we measured accurately the induction of the p19 promoter by Ad infection and Rep expression, and the range from basal to fully induced was almost 3 logs.

The RBE is not a typical upstream activation signal.

We initially expected that Rep would activate the p19 promoter in the same way that its homologue in the autonomous parvovirus family transactivates the p38 capsid promoter. The MVM Rep analog, NS1, has been shown to induce transcription from the p38 capsid promoter by binding to a nearby NS1 binding site and interacting with an Sp1 -50 site upstream of p38 (94, 113, 114). In the case of NS1, it is clear that a C terminal activation domain is essential for induction (106) and that NS1 binding sites behave like upstream activation signals.

To test this possibility for Rep, the RBE was placed at several positions upstream or downstream of an artificial p19 promoter that contained the key p19 promoter

elements required for Rep activation (Fig. 8). However, surprisingly, the RBE resulted primarily in repression of the p19 promoter, and in general, repression was most severe when the RBE was closest to the p19 start site. Several points are worth noting about this experiment. First, in the presence of adenovirus infection, Rep was not capable of transactivating the p19 promoter when only the RBE was inserted into the construct. This clearly showed that the RBE-Rep complex alone was not sufficient for transactivation. It is also consistent with the fact that, to date an activation domain has not been found in Rep. Only the C terminal end of Rep has been found to activate transcription in two hybrid experiments and this region, which is absent in Rep68, is not essential for p19 or p40 activation (29, 202, 203).

The second point worth noting is that the level of repression depended on the conditions of measurement and the distance of the RBE from the start of the p19 mRNA. When Ad infected cells were compared with cells infected with Ad and expressing Rep, the repression was modest, at most 4 fold (in the case of the -145 position). When Rep was expressed by itself (in the absence of Ad) there was a 3-7 fold induction of p19 activity (except in the case of the -100 construct). However, it was clear that placement of the RBE close to the p19 promoter had a severe effect on p19 activity. This was true even in the absence of Rep expression when Ad was present (Fig. 8, Ad only lanes), suggesting that a cellular factor as well as Rep were able to interact with the RBE to cause repression. When the RBE was inserted at the -100 position within the p19 promoter, a region previously shown to have no effect on transcription (144), p19 activity was lower than the basal level under all conditions tested.

Repression by Rep protein has been demonstrated for a variety of heterologous promoters as well as the endogenous AAV promoters. These include the SV40, c-ras, c-myc, c-fos, HPV, HIV and Ad early promoters (7, 8, 66, 74, 82, 91, 97, 143). The mechanism of repression is not clear. Rep has been shown to interact with TBP (69), and one report suggests that Rep may prevent assembly of TFIID complexes on DNA (181). Rep also has been shown to bind to the transcriptional activators PC4 (202), Sp1 (68, 145) and Topors (201), and to the protein kinases PKA and PrKX (29, 40). Recently, Cathomen et al. (21, 22) identified a cellular transcription factor ZF5 that binds to the RBE and may contribute to the cellular repression of p19 seen here in the absence of Rep. Rep has also been shown to stimulate some promoters, for example c-sis and CMV (210, 211). At least two mechanisms appear to be involved in Rep mediated repression as first suggested by Kyostio et al. (98). The first mechanism involves binding of Rep to an RBE as in the case of the p5 promoter. The second does not require an RBE but does require a functional ATP binding site in Rep. Presumably, the ATP binding site is needed either to modify the structure of Rep protein-DNA complexes or to modify the transcriptional template via the Rep associated DNA and RNA helicase activities (75, 209). Consistent with the helicase idea, mutations in the Rep protein's helicase motifs have been shown to be negative for transactivation and repression (98, 124).

The p5 RBE is probably an architectural element designed to bring the p5 and the p19 promoters together.

Our failure to induce p19 activation in artificial constructs that contained only an upstream RBE suggested two other alternatives, read through activation from the p5 promoter and activation by other elements present in the p5 promoter via a looping mechanism. We found no compelling evidence of read through activation from the p5

promoter (Figs. 9 and 10). However, several experiments suggested that the p5 RBE served as a scaffold to bring the p5 promoter elements to the p19 promoter.

Previous work had shown that if purified Rep and Sp1 are incubated with DNA containing the p5 and p19 promoters, a DNA loop can form between the two promoters via Rep-Sp1 interaction (144). In this report, we showed that both the p5 RBE and the p19 Sp1-50 site can be substituted with GAL4 sites. When this was done, activation of the p19 promoter no longer required Rep but depended instead on the presence of hybrid GAL4 fusion proteins containing p53 and T antigen interaction domains. Neither fusion protein contained an activation domain of its own, and neither protein could activate p19 on its own. However, together the two fusion proteins activated p19 transcription nearly as well as Rep and Sp1 (Fig. 11). This demonstrated that the role of the Rep-Sp1 interaction was probably to bring transcription complexes assembled on the p5 promoter to the p19 promoter.

Mutagenesis of the p5 promoter identified at least one element essential for p19 activation, the YY1-60 site. Mutation of this element eliminated activation by the GAL4 hybrid proteins (Fig. 12). In contrast mutation of the p5 MLTF site did not eliminate activation by the hybrid proteins although it did reduce the level of activation. Our experiments did not exclude the possibility that the YY1+1 site or the TATA site was also involved in activation. However, the combination of mutants in YY1-60 and MLTF or YY1-60, MLTF, and YY1+1 were not substantially different the YY1-60 mutant by itself.

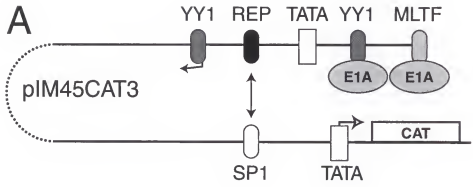
In addition to eliminating p19 activation by Ad and the hybrid proteins, mutation of the YY1-60 site also produced a substantial increase in basal p19 expression and Ad

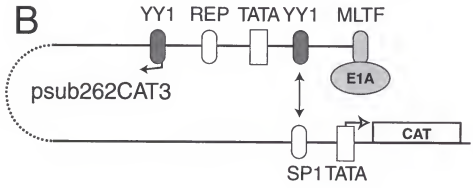
induced p19 expression (Fig. 12). The implication of this is that there are alternative Rep independent interactions between the p5 and p19 promoters and one of these is mediated by the YY1-60 site in the p5 promoter. The net effect of this interaction is to repress p19 promoter basal and Ad induced activity. Thus, the p5 YY1-60 site represses both the proximal p5 promoter and the downstream p19 promoter.

Comparison of the YY1-60 mutant with the parental 2xGAL4 plasmid illustrates one reason for the complexity of AAV transcriptional regulation (Fig. 12). Elimination of this site by mutation increases p19 activity in the presence of Ad to the level seen in the parental plasmid in the presence of Ad and the hybrid factors. Thus, p19 could be induced to the level needed during AAV replication without the need for further activation mechanisms (such as Rep) if the YY1-60 site were not present. However, as mentioned above, elimination of the YY1-60 also increases the basal level of p5 transcription and reduces the Ad induced level of p5 (168). Increasing the basal level of p5 transcription would lead to a virus that is less likely to maintain the latent state because of inappropriate p5 Rep expression. Decreasing the Ad induced level of p5 transcription would reduce the level of AAV DNA replication during productive infection.

To illustrate how p5/p19 interactions might promote activation or repression of p19 under different conditions, we have drawn the model in Fig. 15. The model compares the interaction in pIM45CAT3, which contains wild type p5 and p19 sequences, with that in psub262CAT3, which has a mutant p5 RBE. In the wild type p5 promoter (pIM45CAT), Rep binds to the p5 RBE and forms a complex with Sp1 bound to the Sp1-50 site in the p19 promoter. This places several potential activation regions

Fig 15. **Model of p19 promoter transactivation by interaction with transcription factor complexes bound to p5**. See text for details.


A
YY1
REP
TATA
YY1
MLTF
E1A
E1A
pIM45CAT3
CAT
SP1
TATA


B
YY1
REP
TATA
YY1
MLTF
E1A
psub262CAT3
CAT
SP1
TATA

including those of YY1, MLTF, and E1a near the p19 TATA box to activate p19 transcription. Meanwhile, within the p5 promoter, Rep binding inhibits p5 transcription either by steric hindrance of basal transcription factors (98), by an enzymatic mechanism involving the Rep ATPase activity (98) or by inhibitory contacts with YY1 or E1a. When the RBE is absent (psub262CAT) or in the absence of Rep gene expression, the interaction between p5 and p19 is either absent or occurs between different proteins, for example, YY1 and Sp1 (163). The YY1-Sp1 interaction places activating p5 complexes in a different position with respect to the p19 basal transcription machinery, which may inhibit p19 rather than activating it. Meanwhile, within the p5 promoter, the absence of Rep binding leads to derepression of the p5 promoter (98, 143). The model does not include the contribution of p300 (103) as well as several other cellular and Ad proteins that are likely to be involved, for example, PC4 (202) and DBP (23). In addition, it is likely that other as yet unidentified cellular factors are involved in the complexes that form on the p5 and p19 promoters. However, the model does illustrate how alternative interactions can be established between the two promoters that lead to repression or activation and emphasizes the role of Rep and YY1. The next logical step will be to determine precisely what is present in the p5/p19 complexes in uninfected and Ad infected cells.

Finally, our data also explains a puzzling observation made by Beaton et al (9). This group replaced the p5 promoter sequences upstream of the RBE (including the TATA, YY1-60, and MLTF sites), with the SV40 ori region containing the promoter elements for early SV40 transcription. Surprisingly, this construct was indistinguishable from wild type AAV, but only if it contained an intact terminal repeat. Deletions of the

terminal repeat that removed the TR Rep binding elements were completely defective for AAV transcription. On the other hand, constructs that contained a wild type p5 promoter showed no effect on transcription when the same TR deletions were analyzed. The results obtained with the SV40-AAV hybrids are entirely consistent with our finding that the p5 YY1 site plays a key role in activation of p19. Because the TRs are redundant activation elements that contain an RBE, they substituted for the YY1 complex that was missing in the SV40-AAV hybrid constructs. However, deletions in the TR that removed the RBE presumably prevented their interaction with the AAV promoters, and thus eliminated AAV transcription. This begs the question as to what transcription elements are present in the TR that can substitute for the p5 elements.

Rep Protein Domains in AAV Transcriptional Regulation

A previous study established that two different Rep mutants were created in this manner and resulted in different phenotypes. One mutant contained a magnesium binding mutation that affected the Rep protein in its trs endonuclease activity (50). The mutation did not affect the ability of the Rep protein to bind the terminal repeat or its helicase activity. However, it was demonstrated that the D412A mutant had a diminished ability to interact with Mg2+ ions and an interaction between the Rep D412A mutant and Mg2+ was essential for efficient trs endonuclease cleavage. This data was the first suggestion that a portion of the active DNA cleavage site required a Mg2+ ion and the D412A amino acid.

The second phenotype identified was the first identified conditional lethal mutation within the AAV-2 Rep protein. The D40A,D42A,D44A mutant was found to demonstrate a 3-log difference in rAAV titer between 32C and 39C. Examination of this

mutant in a replication assay showed a fairly strong delay in DNA replication at the permissive temperature of 32C (50) (Lackner in prep). However, the ts Rep mutant showed a complete lack of activity at 39C. In order to better understand the exact alterations in the D40A,D42A, D44A mutant, we analyzed each of the biochemical activities within this protein. We discovered that the temperature sensitivity was mainly dependent on a defect in DNA binding (Lackner in prep). Alteration of the protein sequence inhibited DNA binding of Rep to a synthetic terminal repeat in the AAV genome. The reduced level of DNA binding resulted in a complete loss of Rep endonuclease activity of the AAV terminal repeat.

An important aspect of this D40A, D42A, D44A mutation could be inferred from its sequence homology with other unrelated proteins such as E. coli DNA J and SV40 T-Antigen (Fig. 16). The AAV-2 Rep sequences from amino acids 38 to 42 have a high degree of homology to the chaperone protein domain of DNA J in E. coli and a similar domain of SV40 T-Antigen (20, 166, 177). Based on this protein sequence alignment, the temperature sensitivity of the mutant Rep protein was a result of the disruption of this potential J domain. Similar to its role in SV40 T-Antigen, the potential J-domain within Rep78 may have a direct and stable interaction with the cellular chaperone protein, Hsp70 (62). The J-domain within most polyoma viruses makes direct contacts with the ATPase domain of Hsp70 (20, 88, 122, 161, 166, 197). It has been established through previous studies that the J-domain within SV40 T-Antigen is critical to SV40 DNA replication (20). T-Antigen mutations that affect the J-domain prevent the T-Antigen transactivation of E2F responsive promoters and their subsequent induction in the progression of the cell cycle (177, 180, 222). These same J-domain mutations were

Fig. 16. **Sequence Diagram of Rep Mutagenesis Results.** See text for descriptions.

Rep 78/52 Functional Map

deficient in their capability to bind to Hsp70. The J-domain on SV40 T-Antigen has been identified as a necessary requirement for nuclear localization (216). Direct interaction of the T-Antigen J-domain with Hsp70, results in complementation of a SV40 T-Antigen mutant containing a nuclear translocation and cellular transformation mutation (81). Although the nuclear localization domain of Rep has been localized to amino acids 483 to 519, this J-domain may explain many of the roles for Rep in DNA replication and modification of the cell cycle (99, 186, 198, 199, 206, 207). In SV40, this region has been shown to function as a molecular chaperone mediating phosphorylation of the T-Antigen associated proteins p107 and p130 (180). This same protein domain in Rep may explain its role in interference with viral replication and oncogenicity in SV40, papilloma, and herpesviruses (65, 90, 100, 217, 218). The most likely explanation of the ability of Rep to inhibit cellular transformation after SV40 coinfection could be through a domain negative phenotype. The ability of T-Antigen to promote oncogenesis depends on two factors, the J-domain and the pRB-binding motif (147). The presence of another J-domain containing protein, Rep78, would act as a domain negative protein during an AAV and SV40 coinfection. The Rep78 protein could possibly interact with p107 and p130 but would be unable to fully overcome the G1 arrest or E2F-repression of the cell cycle promoters as a result of its inability to bind pRB. However, in regards to the D40A,D42A,D44A replication mutant, this targeted mutation may disrupt both DNA binding and direct assembly of replication complexes on the AAV terminal repeats. We have demonstrated that the mutation directly inhibits DNA binding and as a result prevents trs endonuclease activity. In addition to its defect in DNA binding, this Rep ts mutant may be defective in its ability to recruit active replication complexes due to this J-

domain mutation. It has suggested that T-Antigen uses its J-domain to support SV40 DNA replication in a manner that is strikingly similar to the use of Escherichia coli DnaJ by bacteriophage lambda in DNA replication (20). In addition to the polyoma virus family, the HPV replication initiator, E1 helicase, has enhanced binding to the origin of replication by the direct interactions of hsp70 proteins (112). This data suggests a strong role for chaperones in viral DNA replication through contacts in a functional J-domain. As result, the inability of this mutant Rep78 to bind the synthetic AAV terminal repeats may be a function of their reduced affinity for the chaperone proteins, Hsp70. Without a possible Hsp70 interaction through the J-domain, the Rep protein complexes may not be correctly assembled for DNA binding and trs endonuclease activity.

The second mutant E345A, E349A affects a possible HMG box in the Rep protein sequence. Between amino acids 340 to 350 of Rep78, protein sequence alignment has identified a possible HMG box that may have multiple roles in the enzymatic activities and biological roles of Rep throughout the AAV lifecycle. Previous research demonstrated that most of the activities associated with the Rep protein are enhanced with the presence and co-expression of the HMG-1 protein (36). This study discovered that the presence of HMG-1 stimulated Rep-mediated trs endonuclease cleavage, ATPase activity, DNA binding, and p5 transcriptional repression. Since the activities of Rep were assayed both in vivo and in vitro, the HMG-1 and Rep protein HMG box may have very similar functional roles in which they recognize the cruciform structures of AAV terminal repeat and form stable nucleoprotein structures. HMG-1 has a general DNA binding activity but an extremely high affinity for cruciform structures and bends in DNA. The sole function of HMG-1 enhancement may be to recognize these odd DNA fragments

within the cell and recruit Rep proteins to form multimeric protein complexes on Rep binding elements. This Rep recruitment would affect every type of RBE within the AAV genome and influence both the latent and lytic lifecycle. The transient transfection of HMG-1 in the presence of a p5 promoter assay was shown to have a stronger level of p5 repression but only in the presence of the Rep protein (36). This data indicates that the assembly of the Rep protein complexes may be more stable in the presence of HMG-1 or may be recruited by HMG-1 bound to the AAV genome.

It was suggested that HMG-1 and HMG-2 interact with transcription factors that contain HMG boxes to stimulate DNA binding and form a DNA bend (184). Transcriptional regulators that contain HMG boxes such as the AAV Rep protein may recruit the HMG-1 protein through protein-protein interactions thereby increasing the affinity of transcription factors to their DNA binding sequence. This increase in transcription factor binding affinity would be dependent on the induction of DNA bending by HMG-1. After the stabilization of transcription factor binding, the DNA bend could recruit a second unrelated transcription factor to another site within the DNA sequence (184). In the case of the E345A,E349A Rep mutant, modification of the HMG box may prevent any possible Rep and HMG-1 protein interaction either in the AAV terminal repeat or the p5RBE. Without the HMG-1 protein to alter the structure of DNA and allow Rep to correctly bind the RBE, a number of biological activities would fail to occur. First, the mutant would have a much lower level of p5 promoter repression as we have shown by transient transfection (Fig. 14, E345A,E349A). The lower level of Rep protein binding would affect both replication and transcription. The defect in p19 transactivation could be the result of failure to produce the proper DNA loop between the

p5 and p19 promoters. If HMG-1 stimulates the Rep protein to tightly bind its RBE, mutation of the HMG box in the Rep protein sequence may prevent a possible p19 transactivation complex. We believe previous studies demonstrating the stable interaction between Sp1 and Rep on AAV support this model (145). However, in vivo conditions may require that the DNA loop created between the p5 and p19 promoter for p19 transactivation be stabilized through the activities of the HMG-1 proteins. This E345A,E349A Rep mutant would support this model because it maintains helicase activities but it is unable to repress or transactivate the AAV promoters (Fig. 13 and 14). Remarkably, the in vitro binding assays between Rep and HMG-1 have indicated that a possible interaction domain between these two protein lies around the proposed Rep HMG box sequence (36).

The next mutant in our charge-to-alanine scanning mutagenesis was the E371A, E372A Rep protein. Although this region of the Rep protein does not contain any known protein motifs, it is in a close proximity to a Rep-Rep interaction domain and the previously discussed HMG box. This mutation caused a replication negative phenotype but did not result in a conditional lethal virus. The E371A, E372A mutant was capable of both DNA binding and helicase activities. However, two very important biological activities were found to be greatly reduced. First, the protein was extremely deficient in its ability to express the Rep 52 and 40 proteins. As further proof for this disruption of the p19 promoter, the pIM45CAT3 reporter construct was unable to transactivate this promoter at a level significantly higher than with Adenovirus alone (Fig. 13). The most interesting effect was the complete lack of transcriptional repression on the p5 promoter. The E371A,E372A mutant was 7-fold higher in its level of p5 promoter expression

compared to the wt level of p5 activity. Interestingly, all of the replication defective mutants had p5 promoter expression that was 3-fold higher than wild type. Although the highest levels of defective p5 promoter repression were focused around the E371A,E372A mutant and the potential HMG box at E345A, E349A (Fig. 13, E345A,E349A and E371A,E372A), other important areas in the Rep protein sequence that targeted specific p5 repression activity colocalized with motifs based on AAV Rep and Rolling Circle Replication (RCR) proteins (Fig. 16). Motif 1 from Rep amino acids 10 to 14 corresponds to the K10A,D14A mutant and motif 2 corresponds with amino acids 90 to 94 similar to the H90A,H92A mutant. Both of these mutations are unable to replicate viral DNA, transactivate the p19 promoter, or repress p5. This combination of mutants would indicate a defect in the assembly of Rep protein complexes similar to the other RCR proteins. The assembly mutants could be unable to tightly bind DNA, have a reduced interaction with possible chaperone activities of HMG-1, or lose the ability to recruit replication machinery to the AAV terminal repeat.

Due to the difficulty in producing large amounts of pure Rep78 for protein crystalization, we are limited in our dissection of the enzymatic activities to protein sequence alignment with other unrelated viral proteins. Based on limited homology with SV40 T-Antigen, previous mutagenesis studies have created ATPase mutants in the Rep protein. However, the elimination of the ATPase function of Rep created a wide variety of lost biochemical activities within the Rep78 protein. The ATPase mutants in those studies also lost DNA helicase activity, the trs endonuclease cleavage at the terminal repeat, and all transactivation from the p19 and p40 promoters (124). Based on homology to those SV40 T-Antigen ATPase mutations, all of the enzymatic activities of

Rep appeared to be dependent on a functional ATPase activity. A new strategy called charge-to-alanine mutagenesis was indiscriminant to helicase homology and targeted only charged amino acid clusters within Rep78 in the hope of creating conditional lethal mutations. Previous work was able to identify an unknown Mg2+ binding site whose mutation resulted in a lack of endonuclease cleavage at the terminal repeat but was completely wild type in other aspects for Rep78 activity. This work has continued to characterize several potential new protein domains within the Rep78 protein. A single HMG box was localized to a specific domain without the loss of any helicase activity and it may have a direct effect in stablizing the DNA loop between the p5 and p19 promoters. Another Rep mutant suggested the role of a J-domain in a temperature sensitive mutant that could influence the ability of the Rep protein in binding to the terminal repeat, trs endonuclease activity, and the recruitment of DNA replication factors at the terminal repeat. However, until the crystalization of Rep78 is performed further localization and biochemical characterization of Rep protein domains will be dependent on continued mutagenesis of the entire protein sequence.

Future Directions

During various cellular conditions, there are a large number of different protein complexes bound to the p5 promoter. One of most important and least examined questions in AAV biology is the actual organization of proteins within the p5 transcriptional complex. One experimental protocol to define the factors bound to this sequence of DNA would be to isolate and purify the p5 promoter. After purification, the p5 promoter element could be incubated in a cellular extract for one of four cellular conditions. The first condition would be a mock extract containing neither an

Adenovirus or Adeno-Associated Virus infection. The second and third conditions would be an single viral infection extract of Ad or AAV, respectively. The last cellular extract would be a combined infection of both Adenovirus and AAV.

With these four types of cellular extract, the p5 DNA sequence could be incubated with them to allow DNA-protein complexes to form on the p5 promoter. After a sufficient time for incubation, the protein complexes formed on the DNA would be fixed by the addition of a reversible cross-linking agent. The DNA sequence could be precipitated after the cross-linking event to isolate the protein complexes. By reversing the mechanism of cross-linking, the DNA-protein complexes could be disassembled and the proteins within this protein complex could be analyzed by various methods. One method of analysis could be a simple Western blot for antibodies of suspected proteins in these complexes. Other methods of analysis could be a 2-D gel electrophoresis or Mass Spectroscopy to gain possible protein sequence information on the proteins precipitated with the p5 promoter DNA.

Another process of p5 promoter transcription complex isolation could be the use of DNA labeled with biomagnetic beads. The purification of the p5 promoter could occur through restriction enzyme digestion. The ends of the restriction enzyme site could be filled by Klenow to incorporate biotinylated labeled dNTPs into the p5 promoter sequence. The biotinylated p5 promoter could be incubated with streptavidin-conjugated Dynabeads. Through the use of a magnet, the labeled p5 promoter could be precipitated, washed, and released from bound DNA-protein complexes after incubation with Ad or AAV infected cells. This biotinylated DNA approach would allow great variety in protein purification by the use of many salt wash conditions. The washed protein

complexes could be purified and analyzed for protein sequence information without reversible cross-linking procedures.

Another protocol for isolating the protein complexes formed on the p5 promoter is the use of DNA affinity column chromatography. The cross-linking of a large number of p5 promoter oligonucleotides to column matrix would enable the formation and purification of proteins bound to the DNA sequence. The four different cellular extracts would be poured over the p5 promoter column and washed with various buffer solutions. The isolation of protein complexes could proceed a number of different ways. One, a reversible cross-linking agent could fix the complexes and later release them after many successive column washings. Second, the washed protein complexes could be fractionated by step or gradient elutions with various salt solutions. Either of these purification protocols would isolate proteins to be sequenced or identified by Western immunoblot procedures.

These three protocols of p5 promoter protein complexes could be analyzed in a number of different ways. First, the structure and composition of these protein complexes could be compared in the four cellular conditions. The composition of these complexes could differ greatly during an Adenovirus infection, Rep protein expression or the combination of both situations. The E1A, E4orf6/7, or any other Adenovirus gene product could influence the structure of these complexes. The stringency of the salt elution or the concentration of cross-linking reagent could determine the overall order of protein binding on the p5 promoter. Second, another interesting assay would be the comparison between the psub262 and p5 promoter. This differential of the two promoters could define the role of Rep in forming various protein complexes as well as

the role of steric interference in p5 transcription factor binding. In combination with the four different cellular extracts, the p5 and psub262 promoters could exhibit and characterize a large number of protein complexes. The analysis of the differential between the mutant sub262 promoter and the wild type p5 promoter in every cellular condition should demonstrate the order of addition in the assembly of transcriptional complexes on the active p5 promoter.

Conclusions

The regulation of gene expression for the Adeno-Associated Virus is one of the most complex and compact transcriptional mechanisms in molecular biology. The entire AAV genome is only 4.5kb in size and contains only three promoter elements. In addition to this relatively minor genome, the lifecycle of AAV can alternate between a latent, repressed proviral state and an actively lytic infection. The regulation for this wide range of transcriptional state is controlled by only one single open reading frame in the AAV genome. The nonstructural proteins of AAV manage every aspect of its lifecycle as well as influence the infected cell environment. These proteins process the cellular stasis of the infected host and decide a wide range of viral responses. If the host cell is healthy, the AAV genome will be directed to site-specifically integrate and repress all viral transcription. If the host cell is coinfected or superinfected with Adenovirus, the AAV genome will respond to the Ad proteins, rapidly replicate its viral DNA, and escape the host cell when the Adenovirus lyzes the host cell. All of these aspects of the AAV lifecycle are processed and directly controlled by the one nonstructural protein, Rep.

The major rate controlled step in the AAV lifecycle is the transcriptional regulation of the p5 promoter. This promoter expresses the largest Rep protein and

determines the rate of Rep and Capsid protein expression. Without Adenovirus, the p5 promoter produces a very minute amount of Rep, which represses the expression of the p5 promoter. However, an Adenovirus infection will induce a large number of changes in p5 transcription. The Early Ad protein, E1A, directly binds to the YY1 repressors on the p5 promoter converting them into activators of Rep expression. The combination of large Rep expression and p5 promoter activation induces the other two promoters of AAV to express their gene products.

The most fascinating AAV promoter is the p19 promoter. The p19 promoter expresses the Rep52 and Rep40 proteins that regulate the expression of the p5 promoter. During Ad infection, the large Rep proteins are expressed from the p5 promoter and inhibit their own expression. In addition to feedback inhibition of the p5 promoter, the large Rep proteins bind the same inhibitory location on the p5 inducing expression from the p19 promoter. The p19 promoter will produce the Rep52 protein which depresses the p5 promoter by directly interacting with the large Rep proteins on the p5 promoter. This regulation and interplay with the p5 and p19 gene products allows the constant expression of large and small Rep proteins. These Rep protein concentrations permit a number of conditions in the lytic phase of AAV. First, the large Rep protein is expressed enough to nick and initiate the replicating pool of AAV genomes. Second, the Rep52 protein concentration is high enough to depress the p5 promoter but low enough to prevent packaging of the entire AAV genome replication pool. Third, the p40 promoter producing Capsid protein is always expressed at excess. The maintenance of this complex transcriptional regulation is controlled by the p5 and p19 promoters.

In previous work, it was shown that Adenovirus always induced high levels of p5 expression. In contrast, the large Rep proteins seemed to exclusively repress the p5 promoter. This data appears opposite to the requirement of Rep for AAV gene expression. This question was addressed in a series of assays to determine if Rep functioned as an enhancer or proximal promoter element.

The Rep binding element was moved to various locations next to the p19 promoter. In the correct genomic location, the p5RBE did not transactivate the p19 promoter. The series of p19CAT3 constructs indicated that as the p5RBE was placed closer to the transcriptional start site, it caused a much higher level of transcriptional repression. These constructs would support the conclusion that the Rep protein does not have any transcriptional activation domain but instead functions exclusively as a transcriptional repressor.

The lack of any transcriptional activation supported the hypothesis that the Rep protein does not exclusively or directly activate transcription. It may function to connect or join two proteins which directly recruit the RNA polymerase II holoenzyme at the p19 promoter. Since the Rep is bound to the p5RBE site in the latent proviral state, the other p5 transcription factors may induce the p19 promoter during an Adenovirus infection. This hypothesis was tested by the subcloning of the p5 promoter elements into the correct spacing in the synthetic p19CAT3 plasmid. With Ad and Rep gene expression, these constructs fail to induce the correct transcriptional regulation. At this point of p19 promoter analysis, it was believed the synthetic nature of the p19CAT3 promoter may lack the correct transcription factor for gene expression. As a result, the CAT reporter gene was subcloned into the AAV genomic subclone plasmid, pIM45.

This pIM45CAT3 plasmid expressed the correct transcriptional regulation with Ad and Rep gene expression. This result implies the sequence of the synthetic p19 promoters cannot exhibit correct p19 gene expression or their Rep-Sp1 interaction is incorrect. The Rep and Sp1 proteins were replaced in the pIM45CAT3 plasmid and replaced with GAL4 hybrid transcription factors. Without any Rep or Sp1 protein sequences, it was shown these two proteins have no direct influence on transcriptional activation of the p19 promoter. The genomic organization of the p5 and p19 promoters form a stable DNA loop through the protein interaction of Rep-SP1 or GAL4 Hybrid transcription factors. This stable DNA loop allows the protein complexes on the Ad-induced p5 promoter to contact and stimulate the formation of a RNA polymerase II holoenzyme at the p19 promoter.

This observation of the p5 promoter transcription factors stimulating the p19 and p40 promoters supports the strong role of p5 induction by the Adenovirus E1A proteins. The mutagenesis of the p5 transcription factors in the GAL4 hybrid reporter plasmid showed several unique facts. First, the mutation of the MLTF site did not affect the Ad and Rep induced transactivation of the p19 promoter. Second, the mutagenesis of both YY1 -60bp and YY1+1 caused the loss of this Ad and Rep induced transactivation. Third, the mutation of the YY1 –60bp induced an extremely high basal transcriptional activity for the p5 promoter. All of these results support the hypothesis that the E1A proteins bound to the two YY1 proteins at the p5 promoter perform the direct transactivation of the p19 promoter.

This series of conclusions has a very profound effect on understanding the basic biology of the Adeno-Associated Virus. First, the nonstructural protein, Rep, exhibits no

transcriptional transactivation activity or domains for the AAV promoters. With or without Ad infection, the large Rep proteins inhibit the transcriptional activity of the p5 promoter. In the context of the p19 promoter, the Rep protein does not transactivate the promoter and will inhibit transcriptional activity when its binding site moved closer to the transcriptional start site. Second, separation of the p5 and p19 promoters by a PolyA Signal influences both a synthetic p19 promoter and the genomic pIM45 backbone. For the genomic plasmids, it strongly decreases the degree of all transcriptional activation. This observation would support the idea that the presence of a terminating RNA polymerase II holoenzyme would inhibit the stable DNA loop formed between Rep and Sp1. The synthetic p19 promoters had a very different result because the lack of a Rep binding element induced the strongest level of Ad and Rep induced transactivation. This effect could be the result of different DNA sequences or the disruption of proper wild type protein-protein interactions. Third, the Rep and Sp1 proteins exhibit no requirement on Ad and Rep induced p19 transactivation. The sole function of these two proteins is to bind DNA sequences at the p5 and p19 promoters and form a stable DNA loop structure. Any other protein complex which performs this same function can replace the role of Rep and Sp1 in AAV transcription. The substitution of GAL4 hybrid transcription factors for Rep and Sp1 proteins supports the hypothesis that the Rep and Sp1 proteins act as architectural proteins in the regulation of AAV gene expression.

APPENDIX
TABLE OF ABBREVIATIONS

AAV	Adeno-Associated Virus
ATP	Adenosine Triphosphate
Ad	Adenovirus
ATPase	Adenosine Triphosphatase
Bp	Base Pair
CAT	chloramphenicol acetyl transferase
CTD	carboxy-terminal domain
DNA	Deoxyribonucleic Acid
Hsp90	Heat Shock Protein 90
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
Inr	Initiator
LCR	Locus Control Region
MVM	Minute Virus of Mice
N-terminus	Amino Terminus
RNA	Ribonucleic Acid
RBE	Rep binding element
TBP	TATA binding protein
YY1	Ying Yang Protein 1
UAS	Upstream Activating Sequence
URS	Upstream Repressing Sequence

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BIOGRAPHICAL SKETCH

Daniel Francis Lackner was born on September 28, 1971 in Midland, Michigan. He spent the first four years of his life in Michigan until his parents moved to Madison, Indiana. He attended Pope John XXII Elementary School and Madison Junior High School in Indiana. While he was fourteen years old, his parents moved back to Midland, Michigan where he finished school at Northeast Junior High School and Midland Public High School. During the fall of 1990, Dan enrolled in college undergraduate studies at Delta College in University Center, Michigan. After the first two years of Delta College, he transferred to Michigan State University in East Lansing, Michigan. He received a Bachelor of Science degree with High Honors in Biochemistry in the spring of 1994. After earning a Biochemistry degree, he obtained a job as a Laboratory Technician at the University of Rochester Biology Department in Rochester, New York. He spent one year in the laboratory of Susan Zusman studying the functions of Integin proteins within the model organism, Drosophilia melanogaster. He began his graduate career by joining the Center of Mammalian Genetics at the University of Florida in the fall of 1995. He joined the laboratory of Nicholas Muzyczka in the summer of 1996. In October of 1999, he married Cari Lynn Aspacher, a fellow graduate student in the Department of Molecular Genetics. In August of 2002, he received a Ph.D in biomedical science.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Nicholas Muzyczka, Chair
Eminent Scholar and Professor of
Molecular Genetics and Microbiology

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Maureen Goodenow
Professor of Pathology, Immunology, and
Laboratory Medicine

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William Hauswirth
Eminent Scholar and Professor of
Molecular Genetics and Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Maurice Swanson
Professor of Molecular Genetics and
Microbiology

This dissertation was submitted to the Graduate Faculty of the College of Agricultural and Life Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Dean, College of Medicine

Dean, Graduate School